INFLAMMATORY MODULATION OF THE RESPONSE OF BRONCHIAL EPITHELIAL CELLS TO LIPOPOLYSACCHARIDE WITH PRETREATMENT BY MONTELUKAST

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ABSTRACT. Montelukast, a leukotriene receptor antagonist, is the most prescribed non-steroidal anti-inflammatory drug used as an add-on therapy for asthma. Besides its effect on blocking leukotriene action, montelukast has been proposed to have secondary anti-inflammatory properties. This study aimed to investigate the modulatory effect of montelukast on the expression of major genes involved in airway inflammation (TNF, IL6) and remodeling (MMP9, TGFBI) in response to lipopolysaccharide (LPS) in vitro. The expression of selected genes was measured by quantitative real-time polymerase chain reaction 0h and 24h after LPS stimulation in cells pretreated with montelukast. Montelukast was found to significantly attenuate increased TNF and IL6 gene expression, to have a mild effect on MMP9 and have no effect on TGFBI expression upon stimulation with LPS. The results of our study indicate that patients on montelukast therapy would have an adequate response to acute microorganism-induced inflammation, so additional anti-inflammatory effects of montelukast should be better exploited.

Keywords: gene expression, inflammation, asthma, montelukast, treatment.

INTRODUCTION

Asthma is a heterogeneous disease, characterized by complex interactions between inflammation and airway remodeling (http://www.ginasthma.org/gina-reports/). The airway inflammation is mediated by a network of mutually interacting cytokines (KIPS, 2001). Among them, interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-α) are considered as important mediators in asthma (BERRY et al., 2007; POYNTER and IRVIN, 2016), since they intertwine in the control of the network of cytokine signaling, inducing different gene expression profiles (GUBERNATOROVA et al., 2021). The major source of constitutively expressed IL-6 in the lungs is the airway epithelium (POYNTER and IRVIN, 2016). Moreover, in response to various stimuli the production of IL-6, as well as TNF-α, is increased in bronchial epithelial cells and inflammatory cells infiltrated in airways such as macrophages, lymphocytes and mast cells (MAHAJAN and MEHTA, 2006; RINCON and IRVIN, 2012; POYNTER and IRVIN, 2016).
The major players in airway remodeling are transforming growth factor beta 1 (TGFβ1) and matrix metalloproteinase 9 (MMP-9) (MATTOS et al., 2002; DUVERNELLE et al., 2003; LI et al., 2007). TGFβ1 is the main profibrotic mediator and its elevated level was observed in asthmatic airways (MASLAN and MIMS, 2014). It promotes subepithelial fibrosis by stimulating the expression of collagen type I and III (MATTOS et al., 2002). MMP-9 represents the major MMP in the airways, and its increased expression is considered as a marker of inflammatory-induced bronchial epithelium damage (OHBAYASHI and SHIMOKATA, 2005; LI et al., 2007).

Montelukast is the most prescribed non-steroidal anti-inflammatory drug used for asthma treatment (JOOS et al., 2008; PAGGARO and BACCI, 2011). It belongs to leukotriene receptor antagonists (LTRAs), the second class of controllers that are used as add-on therapy to inhaled corticosteroids (ICSs) for the achievement of asthma control (BISGAARD, 2000; CAO et al., 2012). LTRAs block specific cysteinyl leukotriene receptors (cysLTRAs) on airway structural and inflammatory cells, thus preventing bronchoconstriction, mucus secretion, and edema in asthmatic lungs. These drugs also reduce the inflow of eosinophils, thereby inhibiting their effects in the airways (TAMADA and ICHINOSE, 2017). However, studies showed that montelukast exhibits additional anti-inflammatory properties unrelated to its action as the antagonist of cysLTRAs (TINTINGER et al., 2010; THERON et al., 2014). It decreases the lipopolysaccharide (LPS)-induced production of TNF-α and IL-6 (HART et al., 1998; MAEBA et al., 2005), and 5-oxo and IL-1β-induced production of MMP9 (LANGLOIS et al., 2006; PIROMKRAIPAK et al., 2018). Additionally, a direct inhibitory effect of montelukast on TGFβ1 gene expression was reported (DRAGICEVIC et al., 2017). On the other hand, the previously observed influence of montelukast on TGFβ1 production was cysLTRAs dependent. The elevated level of TGFβ1 induced by cysteinyl leukotrienes was significantly decreased in response to treatment with montelukast (PERNG et al., 2006). These findings suggest that montelukast could be used as an anti-fibrotic therapeutic agent.

Although studies suggested that montelukast could be used at earlier stages of asthma to prevent disease progression, this drug is not preferred as initial therapy (NAGAO et al., 2018). Together with the direct effects of LTRA, alternative anti-inflammatory and anti-fibrotic properties of montelukast would be useful in clinical practice. Therefore, to fully exploit the benefits of this drug, the extent of its alternative properties should be characterized better.

The aim of this study was to investigate in vitro the modulatory effect of montelukast on the expression of genes associated with airway inflammation and remodeling in response to LPS.

**MATERIALS AND METHODS**

**Cultivation and treatment of BEAS-2B cells**

BEAS-2B, immortalized cell line derived from the human bronchial epithelium, was kindly provided (2014) by Dr. Peter Lackie (School of Medicine, University of Southampton, Southampton, UK). The state of the cells was regularly evaluated by morphology checks using a microscope and performing assays for detection of mycoplasma contamination. The cells were cultivated at 37°C in a 5% CO2 humidified atmosphere in an LHC-9 medium (Thermo Fisher Scientific, USA) supplemented with fetal bovine serum (2%), penicillin (10 U/µL) and streptomycin (10 ng/mL) and subcultured after reaching 70–80% confluence.

For treatment, BEAS-2B cells were seeded in 60 mm cell culture dishes at a density of 8x10⁵/mL. The next day, cells were serum-starved for 1h and treated with montelukast (100 µM). After 1h, LPS (10 µg/mL) was added to cells to induce inflammation. The cells were
stimulated for 2h at 37°C and: 1) immediately collected and lysed for RNA extraction or 2) medium was changed, the cells were incubated for 24h, collected and lysed. The experiment was performed in triplicate and it included untreated cells as control, cells treated with montelukast with and without LPS stimulation, and LPS stimulated cells without montelukast pretreatment.

**Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)**

Cell lysis and total RNA extraction were performed by TRI Reagent Solution (Invitrogen, USA) according to the manufacturer’s protocol. The RNA concentration and quality were determined spectrophotometrically using NanoVue (GE Healthcare Life Sciences, Austria). Reverse transcription was performed using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer’s protocol.

The expression of target genes was measured by quantitative Real-time Polymerase Chain Reaction (qRT-PCR) using 5xHOT FIREPol Eva Green qPCR Mix Plus (ROX) (Solis BioDyne, Estonia). Melting curve analysis was performed after each measurement to confirm the specificity of the fragments. The glyceraldehyde-3- phosphate dehydrogenase (GAPDH) was used as the endogenous control for all measurements. The sequences of primers used to replicate the transcriptional portions of all genes are following: MMP9 forward 5′-GCTCACCTCAGGCTGTA-3′, MMP9 reverse 5′-TCCGTGCTCCCGCACA-3′; TNF forward 5′-CCCAGCGAATCAGGCTCAGAATCTCTTC-3′, TNF reverse 5′-AGCTGCCGCCAGCTTTGA-3′; TGFβ1 forward 5′-AAGGACCTCAGGCTGGAAGTG-3′, TGFβ1 reverse 5′-CCGGTTATGCTGGTGTACAG-3′, IL6 forward 5′-GCAAGGAGCACTGCAGGAAAGTG-3′, IL6 reverse 5′-GGCAAGTCTCCTCATTGAATCC-3′; GAPDH forward 5′-GTGAAGGTGCAGATCAGTAACG-3′, GAPDH reverse 5′-TGAGGTCAATGAAAGGGTCT-3′.

qRT-PCR was performed using 7500 Real-Time PCR System (Applied Biosystems, USA). Thermocycling conditions were the following: initial activation 95°C/12 min, 40 cycles: denaturation 95°C/15 s, annealing 60°C/20 s, elongation 72°C/20 s. All measurements were performed in triplicate and relative quantification was calculated with the ΔΔCt method. The gene expression of GAPDH was used to normalize the relative expression of target genes, while the expression of genes in control untreated cells was used as the calibrator.

**Measurement of Total TGFβ1 by ELISA**

Total TGFβ1 levels were measured in medium collected after treatments of BEAS-2B cells, using the ELISA Multispecies TGFβ1 kit (Invitrogen, USA). The absorbance was read spectrophotometrically on an Infinite 200 PRO multiplate reader (Tecan, Switzerland), using 450 nm and 620 nm filters. Protein concentration (pg/mL) was determined relative to the standard curve and the results were multiplied by the dilution factor (2.2).

**Statistical analysis**

The results were processed in Statistical Package for Social Science (SPSS, USA) software version 25.0. All data were presented as the means ± standard deviation (SD). The Kruskal Wallis test was used to determine significance between all samples. Differences between two independent samples were analyzed using the Independent Samples Mann Whitney U test followed by Benjamini-Hochberg correction. A P value ≤ 0.05 was considered statistically significant.
RESULTS AND DISCUSSION

In order to investigate the modulatory role of montelukast on BEAS-2B cells’ inflammatory response to LPS stimulation, the expression of genes involved in airway inflammation (TNF and IL6) and remodeling (TGFBI and MMP9) was evaluated. Cells were pretreated with montelukast and then stimulated with LPS. The measurement of gene expression was performed at two-time points; upon completion of LPS stimulation (first time point) and 24 h after that (second time point). The results showed that montelukast had no statistically significant effect on the expression of the TNF gene in comparison to untreated cells at both time points (p>0.05) (Fig. 1-1).

Figure 1. Effect of montelukast pretreatment on the expression of genes involved in the inflammatory response. BEAS-2B cells were subjected to indicated agents. Analysis of TNF (1) and IL6 (2) gene expression at the mRNA level was performed at two-time points after LPS stimulation. GAPDH gene expression was used as endogenous control (representative of n = 3) and gene expression in untreated control cells was used as a calibrator. Differences between the two treatments were analyzed by the Independent Samples Mann Whitney U test. Obtained p values were evaluated by the Benjamini-Hochberg method. Results are presented as mean ± standard deviation. * p<0.05; TNF – Tumor necrosis factor; IL6 – interleukin 6; MNT – Montelukast; LPS – Lipopolysaccharide.
As expected, LPS significantly increased TNF gene expression relative to untreated controls, with a stronger effect at the first time point (p=0.01) in comparison to the second (p=0.029). It was observed that increased expression of the TNF gene in response to LPS was reduced in cells pretreated with montelukast at both time points (p=0.01 and p=0.016, respectively).

The expression of the IL6 gene was significantly increased in response to montelukast relative to untreated controls, with a more prominent effect of montelukast observed at the first (P=0.008) than at the second time point (p=0.029) (Fig. 1-2).

![Figure 2](image)

Figure 2. Effect of montelukast pretreatment on the expression of genes involved in the airway remodeling. BEAS-2B cells were subjected to indicated agents. Analysis of MMP9 (1) and TGFB1 (2) gene expression at the mRNA level was performed at two-time points after LPS stimulation. GAPDH gene expression was used as endogenous control (representative of n = 3) and gene expression in untreated control cells was used as a calibrator. Differences between the two treatments were analyzed by the Independent Samples Mann Whitney U test. Obtained p values were evaluated by Benjamini-Hochberg method. Results are presented as mean ± standard deviation. * P<0.05;

MMP9 – Matrix metalloproteinase 9; TGFB1 – transforming growth factor beta 1; MNT – Montelukast; LPS – Lipopolysaccharide.
LPS significantly increased IL6 gene expression compared to untreated controls, with a stronger effect at the first vs. the second time point (p=0.008 and p=0.029, respectively). The LPS-induced increase of IL6 gene expression was reduced in cells pretreated with montelukast at the first time point (P=0.001), as well as at the second time point (p=0.029). The inhibitory effect of montelukast was more pronounced at the second time point.

The expression of the MMP9 gene was slightly increased in cells treated with montelukast compared to untreated controls at both time points, but the difference was not statistically significant (p>0.05) (Fig. 2-1). In response to LPS, MMP9 gene expression was significantly increased only at the second-time point (p=0.029). This increase was reduced in cells pretreated with montelukast compared to cells treated with LPS, but statistical significance was not achieved (p>0.05).

The TGFB1 gene expression upon treatment with montelukast was slightly decreased at the first time point, but this change was not scored at the second time point (Fig. 2-2). Stimulation with LPS initially induced a decrease in TGFB1 gene expression, while an increase in TGFB1 gene expression was noticed after 24 h. However, neither of the findings was statistically significant (p>0.05). LPS-induced changes in TGFB1 gene expression were not significantly affected by montelukast at either of the time points (p>0.05).

We found no significant differences in TGFB1 levels between treatments at the first time point. The concentrations of protein were in the range of 87.6-135.8 pg/mL. At the second time point the concentrations of protein were in the range of 51.6-218.9 pg/mL and 3.3-fold decrease of TGFB1 level upon treatment with montelukast in comparison to untreated control was found (51.6 ± 34.9 pg/mL vs. 172.1 ± 72.2 pg/mL, respectively), while LPS did not influence the production of TGFB1 (201.9 ± 12.9 pg/mL).

The purpose of this study was to explore the potential of montelukast to be used during the initial steps of asthma therapy to prevent inflammation and remodeling, and not only as a supplement to ICSs in the later stages of the disease. The aim of our study was to investigate if the use of montelukast as a pretreatment could alleviate induced inflammation in bronchial epithelial cells. We examined the modulatory effect of montelukast on the expression of major genes involved in inflammation and remodeling in response to LPS using immortalized bronchial epithelial cell line BEAS-2B. This cell line exhibits conserved morphological and functional characteristics encountered in healthy lungs and represents a suitable model system for studying the effects of various environmental factors (ZHAO and KLIMECKI, 2015).

Since microorganisms come into direct contact with the lung epithelium and are the most common cause of inflammation and exacerbations in asthmatics, in our experimental setup we shortly stimulated the cells with LPS to induce acute inflammation. Cells were treated with LPS at a concentration of 10 µg/mL which was previously shown not to have a toxic effect on BEAS-2B cells after 48 h of incubation (VERSPOHL and PODLOGAR, 2012). The expression of TNF, IL6, MMP9, and TGFB1 genes was analyzed at the mRNA level, at two-time points, to elucidate short-term and long-term effects of the acute inflammation. Pretreatment with montelukast was performed to understand how patients on montelukast therapy would react to acute respiratory infections.

The influence of montelukast on the expression of selected genes was examined to understand its effects on healthy lung tissue and how long they last. Our results showed that montelukast initially increased the expression of IL6 genes. However, this effect of montelukast was not long-acting. The observed alterations in the expression of TNF, MMP9 and TGFB1 genes in response to montelukast were not significant. The initial decrease of TGFB1 gene expression agrees with our previous finding (DRAGICEVIC et al., 2017). The direct influence of montelukast on productions of TNF-α, IL-6 and MMP9 was observed in previous studies (HART et al., 1998; MAEBA et al., 2005; LANGLOIS et al., 2006; PIROMKRAIPAK et al.,...
Montelukast decreases its elevated production only after stimulation with leukotrienes (PERNG et al., 2006). Taking this into consideration, we carried out an ELISA test to investigate the effect of montelukast on TGFβ1 production, independently of its function as a cysteine leukotriene (cysLTR) antagonist. Our results showed a 3.3-fold decrease of TGFβ1 at the second time point in response to montelukast, arguing that montelukast by itself inhibited the production of TGFβ1. Since TGFβ1 is released from different cells in latent form, the influence of montelukast on the TGFβ1 activation should be examined in future studies. It could be suggested that the influence of montelukast on the expression of all analyzed mediators most likely involves various mechanisms.

Acute inflammation was induced by LPS. The main mechanism of LPS action includes activation of PI3K/Akt pathway and consequently activation of NF-κB (LIU et al., 2017). Additionally, LPS promotes inflammation in lung epithelial cells by activating MAPK signaling pathway (TSAI et al., 2017). As expected, in our study the expression of pro-inflammatory genes was significantly increased after stimulation with LPS at both time points. A more pronounced effect of LPS on MMP9 gene expression was observed 24 h after stimulation and this finding is in accordance with the literature (TIAN et al., 2018).

The influence of montelukast pretreatment on the gene expression in BEAS-2B cells in response to LPS stimulation was studied to explore the potential of better exploiting this drug in clinical practice. The results showed that montelukast significantly attenuates the LPS–increased TNFα and IL6 gene expression, at both time points. Although montelukast alone induced expression of IL6 gene, it still had an inhibitory effect on LPS-induced IL6 expression, and this effect was long-term. Our results agree with a previous study showing that montelukast reduces TNF-α and IL-6 production upon LPS stimulation, in the culture fluid of the peripheral blood mononuclear cells (MAEBA et al., 2005). This study also proposed that montelukast prevents nuclear translocation of NF-κB induced by LPS.

We observed that montelukast had a long-lasting inhibitory effect on the LPS–increased MMP9 gene expression, although statistical significance was not achieved. In addition to its negative regulation of NF-κB, montelukast was suggested to affect PI3K/Akt and MAPK signaling pathways by reducing the phosphorylation of Akt kinase, MEK and Erk (OJANIEMI et al., 2003; WU et al., 2015). The modulatory effect of montelukast on MMP9 gene expression might be due to the negative regulation of these mediators. On the other hand, slight LPS–induced changes in TGFβ1 gene expression and TGFβ1 production were not significantly affected by montelukast.

Considering that IL-6 produces local inflammatory responses, recruits leukocytes and induces antibody production, and that TNF-α induces pulmonary interstitial edema, our results suggest that patients on montelukast therapy might be more protected from acute inflammation induced by respiratory infections. In the same direction, studies have shown that combined targeting of TNF-α and IL-6 could be more effective than individual inhibition of these cytokines, since it is inhibiting multiple pathogenic mechanisms (GUBERNATOROVA et al., 2021), showing the advantage of montelukast therapy. Patients on this therapy could also be potentially protected from airway damage caused by MMP9. Patients with persistent asthma who do not respond to standard therapy with ICSs might benefit from these alternative properties of montelukast as well (FOGLI et al., 2013).

Therapeutic strategy in asthma tends to reduce ICSs doses due to their adverse systemic effects and find drugs that can compensate for these deficiencies (SCAFFE et al., 2013). Since montelukast has much less harmful effects than steroid drugs, it represents a promising candidate. However, its anti-inflammatory and anti-fibrotic potential has not been fully utilized in therapeutic approaches. Results from our study indicate that montelukast is potentially useful to alleviate inflammation in lung cells, but that it should be carefully dosed so as not to induce gene expression that would accelerate inflammatory processes. The discovery of all the mediators that could be modulated by montelukast would be of great
importance for personalized therapy, especially for the treatment of the patients with increased levels of these mediators in serum.

CONCLUSION

The results of our study suggest that patients already using montelukast therapy would have an adequate response to acute microorganism-induced inflammation. Identification of pro-inflammatory mediators that are modulated by montelukast might lead to more effective therapeutic protocols in patients in whom these mediators are elevated. Overall, our study supports the view that montelukast should be better exploited in clinical practice due to its additional anti-inflammatory effects. However, to elucidate its effects on the expression of different mediators at mRNA and protein levels, clinical studies are needed. Since bronchi-veolar lavage fluid and induced sputum represent a source of various inflammatory cells and soluble compounds, the expression of inflammatory and fibrotic mediators should be analyzed in these samples taken from patients on montelukast monotherapy. Additionally, to clarify the influence of clinically relevant dose of montelukast on the lung epithelium, further studies should be conducted with co-culturing different cell types (e.g., BEAS-2B and eosinophils) or growing BEAS-2B cells in air-liquid interfaces since submerged cultures do not reflect multicellular complexity, differentiation and other airway epithelium characteristics allowing them to differentiate. If montelukast is confirmed to show better effects when used in the initial stages of the disease, it could significantly improve the therapeutic approaches in asthma.

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References:


Pediatric Allergy, Immunology, and Pulmonology 30: 239–245. doi: 10.1089/ped.2017.0770


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