Effects of montelukast on mRNA expression of MUC2 and MUC5B in human nasal epithelial cells

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In this study, the effects of leukotriene receptor antagonist on mRNA expression of MUC2 and MUC5B in human nasal epithelial cells were investigated. Human nasal epithelial cells were randomly assigned into 4 groups: control group, IL-1β group, montelukast + IL-1β group and montelukast group. After 24 h of treatment, the mRNA expressions of MUC2 and MUC5B were determined by fluorescent quantitation PCR. The mRNA expression of MUC2 in the control group, IL-1β group, montelukast + IL-1β group and montelukast group was 2.93±1.57×10^6 copies/µg, 6.63±1.73×10^6 copies/µg, 3.48±1.41×10^6 copies/µg and 1.63±0.47×10^6 copies/µg, respectively (P<0.01). The MUC2 level in montelukast + IL-1β group was lower than in IL-1β group (P<0.01), but there was no marked difference between montelukast group and control group. The mRNA expression of MUC5B in control group, IL-1β group, montelukast + IL-1β group and montelukast group was 8.21±3.54×10^5 copies/µg, 3.40±2.79×10^7 copies/µg, 1.75±0.69×10^6 copies/µg and 5.15±2.16×10^5 copies/µg, respectively, showing significant difference among these groups (P<0.01). In addition, the MUC5B level in montelukast + IL-1β group was lower than in IL-1β group (P<0.05), but there was no marked difference between the montelukast and the control group. Montelukast, a leukotriene receptor antagonist, can decrease the mRNA expressions of MUC2 and MUC5B in IL-1β treated nasal epithelial cells. Our results reveal montelukast may inhibit the inflammatory cytokine induced expression of mucins in nasal epithelial cells.

Key words: Montelukast, leukotriene, MUC2, MUC5B, human nasal epithelial cells.

INTRODUCTION

Leukotrienes (LTs) are pro-inflammatory lipid mediators synthesized in cells from arachidonic acid by 5-lipoxygenase, including LTA4, LTB4, LTC4, LTD4 and LTE4. LTs are secreted mainly by several types of cells including mast cells, eosinophils, macrophages and monocytes. LTs are made from arachidonic acid by the 5-lipoxygenase (5-LO) pathway. As 5-LO is expressed primarily by leukocytes, these cells are the major producers of LTs. There are two types of LTs, LTB4 and the cysteinyl LTs, LTC4, LTD4, and LTE4. LTB4 is recognized as a pro-inflammatory mediator as it attracts and activates a broad array of leukocytes, including monocytes, macrophages and lymphocytes. The cysteinyl LTs are best known for their roles in allergic responses and asthma, where they modulate epithelial, endothelial, and vascular smooth muscle cells. Less well known are the effects of LTs on gene expression. LTB4 alters gene expression on leukocytes and other cell types bearing LTB4 receptors and the cysteinyl LTs modulate gene expression on epithelial, endothelial, and vascular smooth muscle cells, as well as other cells.

The effects of LTs on gene expression are diverse and cell specific, leading to an array of changes that promote inflammation, alter growth rate, and drive tissue remodeling (Yopp et al., 2003; Christmas et al., 2002). Currently, there are three leukotriene receptor antagonists (LTRAs): Montelukast, Pranlukast and Zafirlukast.

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Montelukast is a highly selective cysteinyl leukotrienes receptor inhibitor and can competitively counteract with the binding of LTD4 to CysLT1. Human CysLT1 is a member of the superfamily of G protein-coupled receptor, consists of 337 amino acids and has a seven-transmembrane domain. The CysLT1 gene is located on Xq13-q21. The affinity of LTD4 to receptors is as follows: TD4>LTC4>>LTE4.

The affinity of LTD4 to CysLT1 is about 50-fold higher than that of LTC4 to CysLT1. CysLT1 is expressed on mast cells, eosinophils, neutrophils, smooth muscle cells and lung fibroblasts (Capra, 2004). In addition, CysLT1 is also detected in gland cells and epithelial cells of sinus mucosa (Corrigan et al., 2005). Studies have demonstrated LTs play important roles in the chronic nasal sinusitis and allergic rhinitis. In these diseases, LTs can promote the edema of nasal mucosa, facilitate the aggregation and activation of inflammatory cells, stimulate the secretion of mucus and compromise the clearance of mucus by bronchial cilia (Peters-Golden et al., 2006; Pérez-Novio et al., 2005). Mucins are the main stimulators of mucus and compromise the clearance of mucus by bronchial cilia. In these diseases, LTs can promote the edema of nasal mucosa, facilitate the aggregation and activation of inflammatory cells, stimulate the secretion of mucus and compromise the clearance of mucus by bronchial cilia. Mucins are the main components of nasal mucus and determinants of gel-like characteristics of mucus.

In the present study, nasal epithelial cells were treated with one of LTRAs aiming to investigate the effects of LTRAs on the mRNA expressions of MUC2 and MUC5B.

**MATERIALS AND METHODS**

**Drugs and reagents**

Fetal bovine serum (FBS; PAA, Austria), collagenase (Cibco), insulin (Sigma), transferring (Sigma, USA), human endothelial cell growth factor (Cytolab Ltd, USA), IL-1β (peprotech), TRIZol (invitrogen) and montelukast (Merck Sharp and Dohme Ltd.) were used in the present study.

**Main instruments**

PE9600 PCR instrument (Perkin Elmer, USA), slide observation machine, Olympus audio-video system (Olympus, Japan), ultraviolet spectrophotometer (SHIMADZU UV mini 1240, Japan), incubator (FORMA 3110, USA), and PE 7000 automatic fluorescent quantitation PCR instrument (Perkin Elmer, USA) were used in this study.

**Culture of nasal epithelial cells**

Informed consent was obtained from patients before study. The mucosa of inferior turbinate was obtained in nasal septum surgery. Patients did not receive glucocorticoids or anti-histamine drugs, were not sensitive to aspirin and had no history of asthma or allergic rhinitis. The nasal epithelial cells were isolated according to previously described (Yamaya et al., 1992). The mucosa was cut into pieces and 0.1% collagenase and 100 µg/ml streptomycin. The tissues were washed with normal saline containing 100 IU/ml penicillin and 100 µg/ml streptomycin. This protocol was repeated until the tissue was completely digested. The collagenase was added at a volume ratio of 1:3 followed by digestion for 30 min at 37°C. Centrifugation was performed at 100 rpm for 5 min and the supernatant was discarded.

The sediment was re-suspended in 3 ml of medium containing FBS and cell suspension was added to flasks followed by incubation in an atmosphere with 5% CO2 at 37°C. One day later, 2/3 of supernatant was replaced with serum-free medium (DMEM-Ham F12 medium) containing 5 µg/ml insulin, 1 µg/ml transferin, 2 µg/ml human epidermal growth factor, 3.75 µg/ml human endothelial cell growth factor, 3.75 µg/ml hydrocortisone, 100 IU/ml penicillin and 100 µg/ml streptomycin. Medium was refreshed two days later, and passing was performed when cell confluence reached near 100%. The medium was discarded and cells were washed with aseptic Hanks solution thrice. Then, 0.25% trypsin (50 µg/ml) was added and cells were observed under a microscope. When contraction was observed in about 90% cells showing cell exfoliation, cells were pipetted and cell suspension was prepared. These cells were seeded in flasks at an appropriate density followed by incubation in an atmosphere with 5% CO2 at 37°C. One day later, the medium was replaced with serum-free medium.

**Treatment of nasal epithelial cells with LTA**

The nasal epithelial cells of passage 2 were stained with trypan blue and the cell viability was greater than 95%. These cells were seeded in 6-well plate at a density of 5×10^5/ml followed by incubation in serum-free medium in an atmosphere with 5% CO2 at 37°C. Medium was refreshed every other day. After 7~8 days of culture, when cell confluence reached nearly 100%, cells were divided into four groups: in IL-1β group, cells were treated with 10 ng/ml IL-1β in serum-free medium (n = 6); in montelukast + IL-1β group, cells were treated with montelukast (10^4 M/ml) for 8 h and then with IL-1β (10 ng/ml) in serum-free medium (n = 6); in montelukast group, cells were treated with montelukast (10^5 M/ml) alone (n = 6); in control group, cells were incubated in serum-free medium (n = 6). One day later, cells were harvested and mRNA expressions of target genes were determined.

**Detection of mRNA expressions of target genes by fluorescent quantitation RT-PCR**

Total RNA was extracted with TRIZol and the concentration of RNA was determined with a UV spectrophotometer. Then, 4 µl of total RNA were used for reverse transcription and the mixture (20 µl) included 4 µl of 5 × buffer, 0.4 µl of forward primer (10 pM/µl), 0.4 µl of reverse primer (10 pM/µl), 0.2 µl of dNTPs (25 mM), 1 µl of MMLV (10 U/µl), 10 µl of DEPC treated water, and 4 µl of total RNA. The 5 × buffer contains 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 4 mM MgCl2, and 10 mM DTT. Reverse transcription was performed at 37°C for 1 h and then at 95°C for 3 min. Then, fluorescent quantitation PCR was performed. The primers and fluorescent probe were synthesized in Da'an Gene Co., Ltd. The primers and probe for MUC2 were as follows:

Forward: 5'-GGT GGA GAC ACA GAA TTG ATT GG-3'; reverse: 5'-FAM-CAC GCC GTT CTT GCT-3'; probe: 5'-FAM-CAA ATT CCG GTG AGC AGC GGT-TAMRA-3'.

The primers and probe for MUC5B were as follows:

Forward: 5'-GGG GGA GCT GTG TGG TTG C-3'; reverse: 5'-AAG CAC GCC GTT CCT GCT-3'; probe: 5'-FAM-CAA ATT CCG GTG AGC AGC GGT-TAMRA-3'
The mixture (50 µl) included 10 µl of 5 x PCR buffer, 1 µl of forward primer (10 PM/µl), 1 µl of reverse primer (10 PM/µl), 0.5 µl of dNTPs (25 mM), 1 µl of probe (10 PM/µl), 1.5 µl of Taq DNA polymerase, 5 µl of cDNA and 30 µl of ddH2O. The PCR buffer consists of 10 mM Tris-HCl (pH 8.0), 50 mM KCl and 2 mM MgCl2. The conditions for PCR were as follows:

40 cycles of denaturation at 93°C for 3 min, annealing at 93°C for 45 s and extension at 55°C for 45 s.

The fluorescence signals of each gene were input into a computer and analyzed by software followed by calculation of copy number and Ct value (the cycle at which the fluorescence from a sample crosses the threshold). According to the RNA concentration, the expressions of MUC2 and MUC5B were calculated (copy/µg cDNA).

Statistical analysis

Data were expressed as means ± standard deviation (±s) and statistical analysis was performed with SPSS version 9.0 statistical software package. Quantitative data had homogeneity of variance demonstrated by test, and analyzed with one way analysis of variance (ANOVA). Comparisons between two groups were done with Student -Newman-Keuls test. A value of P<0.05 was considered statistically significant.

RESULTS

Amplification curves and standard curve

In the fluorescent quantitation, the standard amplification curve of MUC2, standard curve of fluorescent quantitation and amplification curve of MUC2 in human nasal epithelial cells were presented in Figure 1A, B and C. The standard amplification curve of MUC5B standard, standard curve of fluorescent quantitation and amplification curve of MUC2 in human nasal epithelial cells are presented in Figure 2 A, B and C.

mRNA expressions of MUC2 and MUC5B in human nasal epithelial cells

The mRNA expressions of MUC2 and MUC5B in different groups were presented in Table 1. The mRNA level of MUC2 in montelukast + IL-1β group was lower than that in IL-1β group (t = 3.44, P<0.01), and significant difference was noted between IL-1β group and control group (t = 3.88, P<0.01). But there was no marked difference in MUC2 expression between montelukast group and control group (t = 1.00, P>0.05). In addition, the mRNA level of MUC5B in montelukast + IL-1β group was lower than that in IL-1β group (t = 2.81, P<0.05) and significant difference was noted between IL-1β group and control group (t = 2.89, P<0.05). No marked difference was noted in MUC5B expression between montelukast group and control group (t = 1.81, P>0.05).

DISCUSSION

Mucins are a family of high molecular weight, heavily glycosylated proteins (glycoconjugates) produced by epithelial tissues in most metazoans. Although some mucins are membrane-bound due to the presence of a hydrophobic membrane-spanning domain, most mucins are secreted onto mucosal surfaces or secreted to become a component of saliva. The amount of secreted mucins determines the viscosity and elasticity of mucus. Airway epithelial cells can express secreted mucins and MUC2 and MUC5B are the main mucins in the airway mucus (Chen et al., 2004). However, the secretion and regulation of mucins are still poorly understood. Study showed IL-β could up-regulate the mRNA expressions of MUC2 and MUC5B (Huang et al., 2010) and Ishinaga et al. (2005) revealed Pranlukast, a LTA, could significantly inhibit LPS induced mRNA expression of MUC2 in NCI-H292 cells. Bai et al. (2007) also noted Pranlukast could markedly suppress LTD4 induced expression and secretion of MUC2 in airway epithelial cells. Montelukast is a highly selective LTRA and can counteract with the binding of LTD4 to CysLT1 receptor.

Montelukast has multiple biological activities:

1) It can improve the hyperresponsiveness. Phipatanankul et al. (2002) showed one week of treatment with Montelukast could significantly improve the hyperresponsiveness in allergic asthma children and prolong the time of tolerance to cat allergens, which suggest Montelukast has the characteristic of improving airway hyperresponsiveness.

2) It can inhibit airway remodeling. Airway remodeling is a result of repeated inflammatory damage and repair and characterized by infiltration of eosinophils in airway, hypertrophy of goblet cells and smooth muscle cells and subepithelial fibrosis. Henderson et al. (2002) reported inhalation of OVA resulted in infiltration of EOS and monocytes, hypertrophy of goblet cells, and deposition of a large number of collagens under epithelial cells. After treatment with montelukast, these pathological features were markedly improved.

3) It can inhibit the migration, aggregation, proliferation and activation of inflammatory cells. Montelukast can decrease the Mac-1 expression and EOS migration induced by CysLTs, inhibit LTD4 induced proliferation and activation of EOS, and decrease the infiltration of EOS in airway (Fregonese et al., 2002).

4) It can inhibit the release of inflammatory mediators and cytokines. Montelukast could decrease the expression of IL-5, IL-13 and vascular cell adhesion molecule in the lung of acute asthma animals, decrease the levels of ET-1 and INF-γ in bronchoalveolar lavage fluid (Wu et al., 2003). These findings suggest Montelukast can inhibit the release of some cytokines and inflammatory mediators.

In the present study, the mRNA expressions of MUC2 and MUC5B were not significantly changed in...
Figure 1. Amplification curves and standard curve of MUC2. A). Standard amplification curve of MUC2 standard (a: 1/2 dilution; b: 1/10 dilution; c: 1/100 dilution; d: 1/1000 dilution); B). The standard curve of MUC2; C) the amplification curves of MUC2 in human nasal epithelial cells.
Figure 2. Amplification curves and standard curve of MUC2. A) Standard amplification curve of MUC5B (a: 1/2 dilution; b: 1/10 dilution; c: 1/100 dilution; d: 1/1000 dilution); B) Standard curve of MUC5B; C) the amplification curves of MUC5B in human nasal epithelial cells.
Table 1. mRNA expressions of MUC2 and MUC5B in nasal epithelial cells (±s).

<table>
<thead>
<tr>
<th>Group</th>
<th>MUC2 (copy/µg)</th>
<th>MUC5B (copy/µg)</th>
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<tbody>
<tr>
<td>Control</td>
<td>(2.93±1.57)x10^6</td>
<td>(8.21±3.54)x10^5</td>
</tr>
<tr>
<td>Montelukast</td>
<td>(1.63±0.47)x10^6</td>
<td>(5.15±2.16)x10^5</td>
</tr>
<tr>
<td>IL-1β</td>
<td>(6.63±1.73)x10^6</td>
<td>(3.40±2.79)x10^7</td>
</tr>
<tr>
<td>Montelukast + IL-1β</td>
<td>(3.48±1.41)x10^6</td>
<td>(1.75±0.69)x10^6</td>
</tr>
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F value: 14.10 8.25  
P value: <0.01 <0.01

Montelukast group when compared with control group, which suggests Montelukast does not affect the expressions of MUC2 and MUC5B in normal nasal epithelial cells. However, the mRNA expressions of MUC2 and MUC5B in Montelukast + IL-1β group was lower than that in IL-1β group (P<0.01 and P<0.05, respectively), which implies Montelukast can inhibit the IL-1β induced expressions of MUC2 and MUC5B. Wilson et al. (2001) applied Montelukast (10 mg/d) to treat chronic rhinosinusitis, and symptoms were significantly improved. Philip et al. (2007) used Montelukast to treat allergic rhinitis achieving good outcome. Our results showed Montelukast inhibited the IL-1β induced expressions of MUC2 and MUC5B in nasal epithelial cells. We speculate LTAs can be used to improve chronic rhinosinusitis and allergic rhinitis through suppressing the expression and secretion of mucins.

REFERENCES


