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Effects of montelukast on subepithelial/peribronchial fibrosis in a murine model of ovalbumin induced chronic asthma

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Montelukast, a leukotriene receptor antagonist, is used commercially as a maintenance treatment for asthma and to relieve allergic symptoms. In this study, we evaluated the protective effects of montelukast against the airway inflammation and fibrosis using a murine model of ovalbumin (OVA) induced chronic asthma. The animals received OVA challenge three times a week for 4 weeks. Montelukast (30 mg/kg) was administrated orally once a day for 4 weeks. The administration of montelukast caused a reduction in elevated interleukin (IL)-4, IL-13, eotaxin, immunoglobulin (Ig), inflammatory cell infiltration into the airways, and mucus production after repeated OVA challenges. To investigate the antifibrotic mechanism of montelukast, we examined the expression of profibrotic mediators, including vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β_1, and Smad3 proteins in the lung tissue using western blotting and immunohistochemistry. The administration of montelukast reduced the overexpression of profibrotic proteins in the lung tissue, which was confirmed by immnunohistochemistry. These results are consistent with a histopathological examination of lung tissue with Masson's trichrome stain. In conclusion, the administration of montelukast reduced airway inflammation and pulmonary fibrosis by reducing the release of Th2 cytokines and the expression of VEGF, TGF-β_1/Smad3 in the lung tissue.

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1. Introduction

Chronic asthma is characterized by chronic inflammation, mucus hypersecretion, and subepithelial/peribronchial fibrosis. It develops when an individual is exposed to various risk factors, such as allergen, drugs, radiation, infection, and toxic particles, and causes pathophysiological changes. Increases in T helper (Th)2 cytokines, such as interleukin (IL)-4, IL-5, and IL-13, are crucial pathophysiological changes in chronic asthma [1,2]. Th2 cytokines are produced by activated Th2 cells, which induce inflammatory cell infiltration into the airways [3]. Th2 cytokines are also associated with several growth factors, including vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-β_1 [1,4], which are important and well-known mediators of airway remodeling processes, including chronic inflammation, mucus hypersecretion, and fibrosis. According to previous studies, TGF-β_1 induces the deposition of collagen, resulting in pulmonary fibrosis [5]. VEGF increases inflammatory cell infiltration into the airways by enhancing vascular permeability and Th2 cytokine release [6]. Recently, Vasquez-Pinto et al. [7] demonstrated that VEGF causes pulmonary fibrosis by increasing the expression of Th2 cytokines and TGF-β_1, and these observations are consistent with the results of Lee et al. [5].

Montelukast, a leukotriene receptor antagonist, is used commercially used as a maintenance treatment for asthma and to relieve allergic symptoms. Montelukast inhibits the action of leukotriene D4 on the cysteinyl leukotriene receptor (CysLTR)1 in the lung, which reduces bronchoconstriction and airway inflammation [8,9]. Previous studies have demonstrated that montelukast attenuates the airway remodeling induced by several allergens by modulating the expression of Th2 cytokines and growth factors [10–12]. In addition to these features, several researchers have demonstrated that montelukast exerts beneficial effects against experimentally induced toxicities. Mohamadin et al. [13] reported that montelukast reduced lipopolysaccharide-induced liver damage via its antioxidant properties. Montelukast also displayed protective effects against experimentally induced renal failure and gastrointestinal injury in many experiments *in vivo* and *in vitro* [14–17].

In this study, we investigated the antifibrotic effects of montelukast in chronic asthma induced by repeated ovalbumin (OVA) challenge. To understand the antifibrotic mechanism of montelukast, we also evaluated the expression of profibrotic mediators, including VEGF, TGF-β_1 and Smad3 using immunoblotting and immunohistochemistry.

2. Materials and methods

2.1. Animals

Specific-pathogen-free female BALB/c mice (7 weeks old) were purchased from the Orient Co. (Seoul, Korea) and used after 1 week of quarantine and acclimatization. The mice were allowed sterilized tap water and standard rodent chow *ad libitum*. All experimental

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procedures were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Chungnam National University Animal Care and Use Committee. The animals were cared for in accordance with the dictates of the National Animal Welfare Law of Korea.

2.2. Experimental procedures

OVA sensitization and airway challenge were performed as described previously [18]. In brief, mice were sensitized on days 0 and 14 with an intraperitoneal injection of 20 μg of OVA emulsified in 2 mg of aluminum hydroxide in 200 μL phosphate-buffered saline (PBS; pH 7.4). One week after the final sensitization, the mice underwent airway challenge with OVA (1%, w/v, in PBS) for 1 h with an ultrasonic nebulizer (NE-U12; Omron Corp., Tokyo, Japan) three times a week for 4 weeks. Montelukast was obtained from the Korea Research Institute of Bioscience and Biotechnology and was dissolved in PBS and was prepared fresh daily before each treatment. It was administered by gavage to the mice at doses of 30 mg/kg once daily for 4 weeks.

Following the OVA challenge, samples of bronchoalveolar lavage fluid (BALF) were collected from the mice and processed, and the inflammatory cells were counted as described previously [2]. In brief, the mice were killed with an intraperitoneal injection of pentobarbital (50 mg/kg; Hanlim Pharmaceutical Co., Seoul, Korea) 48 h after the last challenge, and a tracheostomy was performed. To obtain the BALF, ice-cold PBS (0.5 mL) was infused three times into the lungs and withdrawn via tracheal cannulation (total volume of 1.5 mL). The total numbers of inflammatory cells were assessed by counting the cells in at least five squares of a hemocytometer after the dead cells were excluded by Trypan blue staining. To prepare the cells for counting, 100 μL of BALF was centrifuged onto slides at 200 ×*g* and 4 °C for 10 min using a Cytospin (Hanil Science Industrial, Seoul, Korea). The slides were dried, and the cells were fixed and stained using Diff-Quik® staining reagent (B4132-1A; IMEB Inc., Deerfield, IL), according to the manufacturer's instructions. The supernatant obtained from the BALF was stored at −70 °C for biochemical analysis.

2.3. Measurement of cytokines, chemokine, and IgE levels in BALF or plasma

The levels of IL-4, IL-13, and eotaxin in the BALF were measured using enzyme-linked immunosorbent assay (ELISA) kits (BioSource International, Camarillo, CA) according to the manufacturer's protocols. The levels of total IgE and OVA-specific IgE in BALF and plasma were measured using an ELISA. Microtiter plates were coated with anti-IgE antibody (anti-mouse IgE; 10 g/mL; Serotec, Oxford, UK) in PBS-Tween 20, and incubated with a BALF or plasma sample. The plate was then washed four times, and 200 μL of o-phenylenediamine dihydrochloride (Sigma-Aldrich, St. Louis, MO) was added to each well. The plates were incubated for 10 min in the dark and the absorbance was then measured at 450 nm. This assay was performed in duplicate.

2.4. Measurement of TGF-β_1 and VEGF levels in lung tissues

The levels of TGF-β_1 and VEGF in the BALF were measured with ELISA kits (R&D Systems, Inc., Minneapolis, MN and Immuno Biological Laboratories Co., Ltd., Minneapolis, MN, respectively), according to the manufacturers' protocols. Total proteins were determined using a protein assay reagent (Bio-Rad, Hercules, CA). The results were expressed as pg/mg protein. This assay was performed in duplicate.

2.5. Immunoblotting

Equal amounts of total lung proteins (30 μg) were heated at 100 °C for 5 min, loaded onto 8% SDS-PAGE gels, and separated electrophoresed. The proteins were then transferred to a nitrocellulose membrane (at 100 V for 2 h), and the membrane was blocked for 1 h with Tris-buffered saline containing 0.05% Tween-20 (TBST) plus 5% skim milk. The membrane was then incubated overnight at 4 °C with anti-VEGF antibody (1:1000 dilution; Abcam, Cambridge, MA), anti-TGF-β_1 antibody (1:1000 dilution; Abcam), anti-pSmad3 and -smad3 antibody (1:1000 dilution; Abcam), and anti-β-actin antibody (1:1000 dilution; Cell Signaling Technology, Danvers, MA). The membrane was washed three times with TBST and then incubated with a horseradish peroxidase-conjugated secondary antibody (1:3000 dilution; Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. The membrane was washed three times again with TBST and developed using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Uppsala, Sweden). For the quantitative analysis, densitometric value of each band was determined using Chemi-Doc (Bio-Rad). This assay was performed in 7 mice per groups.

2.6. Histology

After the BALF samples were collected, the lung tissue was fixed in 10% (v/v) neutral-buffered formalin. The tissues were embedded in paraffin, sectioned to 4 μm thickness, and stained with hematoxylin and eosin (H&E) solution (hematoxylin, Sigma MHS-16; eosin, Sigma HT110-1-32) and periodic acid-Schiff (PAS; IMEB Inc., San Marcos, CA) to estimate inflammation and mucus production, respectively. To evaluate peribronchial fibrosis, paraffin-embedded tissue was stained with Masson's trichrome. Quantitative analysis of inflammation, mucus production, and collagen deposition was determined using an image analysis (Molecular Devices, Inc., CA, USA). Slides were prepared for two per animal (n = 7/group). In quantitative analysis, we randomly chosen five histological fields per slide focused on bronchial (×200 magnification).

For the immunohistochemical analysis, paraffin sections were deparaffinized, dehydrated, washed in PBS containing 0.3% Triton X-100, and preincubated for 10 min at room temperature with 10% goat serum to block nonspecific staining. The slides were then incubated overnight at 4 °C with primary mouse anti-rabbit VEGF antibody (1:200 dilution; Abcam) and TGF-β_1 (1:1000 dilution; Abcam). After the primary antibodies were removed, the sections were washed and incubated at 37 °C for 1 h with biotinylated secondary antibody, and then incubated with an avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Excess complex was removed, and the sections washed with PBS and incubated with 0.05% diaminobenzidine (1:200; Millipore, Billerica, MA) for a further 10 min. The sections were counterstained, rinsed in PBS to terminate the reaction, and protected with coverslips for microscopic examination. This assay was performed in all mice of each group.

2.7. Image capture and photomicrography

Photomicrographs were taken with a Photometric Quantix digital camera running a Windows program, and montages were assembled in Adobe Photoshop 7.0. The images were cropped and corrected for brightness and contrast, but were not otherwise manipulated.

2.8. Statistical analysis

Data are expressed as means ± standard errors of the mean (SEM). Statistical significance was determined using analysis of variance (ANOVA) followed by a multiple comparison test with Bonferroni adjustment. P values < 0.05 or < 0.01 were considered significant.

3. Results

3.1. Effect of montelukast on the number of inflammatory cells in BALF

The numbers of inflammatory cells in the BALF were significantly increased in the OVA-challenged mice compared with those in the

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