

Montelukast prevents the decrease of interleukin-10 and inhibits NF- κ B activation in inflammatory airway of asthmatic guinea pigs

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Abstract: Interleukin (IL)-10 is an important immunoregulatory and anti-inflammatory cytokine, whereas nuclear factor- κ B (NF- κ B) plays an important role in the pathogenesis of asthma. In the present study, the effects of montelukast on the level of IL-10 and on the activation of NF- κ B in the inflammatory airway of asthmatic guinea pigs were investigated. Guinea pigs were sensitized by ovalbumin. Pulmonary inflammation was observed by hematoxylin and eosin staining. The eosinophils in broncho-alveolar lavage fluid and blood were separated by density gradient centrifugation and counted under microscope. The level of IL-10 in broncho-alveolar lavage fluid was measured by enzyme-linked immunosorbent assay. Activation of NF- κ B in lung tissues was inspected by immunohistochemistry. Montelukast at medium and high doses prevented the decrease of IL-10 in broncho-alveolar lavage fluid ($n = 8$, $p < 0.01$ vs. asthma model group), inhibited the activation of NF- κ B in lung tissues ($n = 8$; medium dose, $p < 0.05$; high dose, $p < 0.01$; vs. asthma model group). There was a significantly negative correlation between the level of IL-10 and the activation of NF- κ B in lung tissues ($r = -0.488$, $p < 0.01$). Montelukast reduced the severity of airway inflammation and the number of eosinophils in asthmatic guinea pigs. From all these findings we conclude that montelukast can prevent the decrease of IL-10 and inhibit the activation of NF- κ B in inflammatory airway of asthmatic guinea pigs, which may be the new important mechanisms of montelukast's anti-airway-inflammation effects in asthmatic guinea pigs.

Key words: montelukast, asthma, airway inflammation, IL-10, NF- κ B.

Résumé : Il est reconnu que l'interleukine (IL)-10 est une cytokine immunorégulatrice et anti-inflammatoire importante, tandis que le facteur nucléaire κ B (NF- κ B) joue un rôle majeur dans la pathogenèse de l'asthme. Dans la présente étude, nous avons examiné les effets du montelukast sur le taux d'IL-10 et l'activation du NF- κ B dans les voies aériennes enflammées de cobayes asthmatiques. Nous avons sensibilisé les cobayes au moyen d'ovalbumine. Nous avons observé l'inflammation pulmonaire par coloration à l'hématoxyline-éosine (HE). Nous avons séparé les éosinophiles dans le liquide de lavage bronchoalvéolaire (LLBA) et le sang par centrifugation en gradient de densité et nous avons dénombrés sous microscope. Nous avons mesuré le taux d'IL-10 dans le LLBA par dosage immunoenzymatique (ELISA). Nous avons examiné l'activation du NF- κ B dans les tissus pulmonaires par immunohistochimie. Des doses moyennes et élevées de montelukast ont prévenu la diminution du taux d'IL-10 dans le LLBA ($n = 8$, $p < 0.01$ vs. groupe modèle d'asthme) et inhibé l'activation de NF- κ B dans les tissus pulmonaires ($n = 8$, $p < 0.05$, $p < 0.01$ vs. groupe modèle d'asthme). Il y a eu une corrélation négative significative entre le taux d'IL-10 et l'activation de NF- κ B dans les tissus pulmonaires ($r = -0.488$, $p < 0.01$). Entre-temps, le montelukast a réduit la sévérité de l'inflammation des voies aériennes et le nombre d'éosinophiles chez les cobayes asthmatiques. À la lumière de ces résultats, nous concluons que le montelukast peut prévenir la diminution du taux d'IL-10 et prévenir l'activation de NF- κ B dans les voies aériennes enflammées des cobayes asthmatiques, ce qui pourrait constituer les nouveaux mécanismes importants des effets anti-inflammatoires des voies aériennes du montelukast chez les cobayes asthmatiques.

Mots clés : montelukast, asthme, inflammation des voies aériennes, IL-10, NF- κ B.

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Introduction

Bronchial asthma is defined as a chronic airway inflammatory disease, characterized by infiltration of eosinophils, lymphocytes, and mast cells. Many cytokines and inflamma-

tory mediators are involved in the condition (Chinen and Shearer 2004). Cysteinyl leukotrienes (cys-LTs), potent proinflammatory mediators, have been recognized as powerful spasmogens for bronchial smooth muscle and thus have been implicated in the pathogenesis of asthma (Leru 2003).

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Cys-LTs can augment both accumulation and activation of eosinophils, increase vascular permeability and mucus secretion, as well as promote smooth muscle proliferation, which contributes to airway inflammation in asthma (Busse 1996; Drazen et al. 1999; Nagata and Saito 2003). Leukotriene receptor antagonists are the new class of asthma therapy agents that selectively block the leukotriene pathway involved in the pathophysiology of asthma (Bisgaard and Nielsen 2000; Knorr et al. 2001). Montelukast is a cysteinyl leukotriene D₄-receptor antagonist. Its anti-asthmatic effect has been confirmed by many studies (Knorr et al. 1998; Reiss et al. 1996; Reiss et al. 1998). However, the detailed mechanisms of montelukast antagonizing airway inflammation of asthma remain to be investigated.

Nuclear factor- κ B (NF- κ B) is a critical transcription factor that regulates the expression of a wide range of genes involved in immune and inflammatory response (Bremner and Heinrich 2002). Interleukin (IL)-10 is an important anti-inflammatory cytokine (Bellingshausen et al. 2001). IL-10 can attenuate the reduction in inhibitory- κ B (I- κ B), inhibit the activation of NF- κ B, and reduce the excessive inflammation (Bhattacharyya et al. 2004; Higuchi et al. 2004). Recent studies demonstrated that montelukast can significantly increase the level of IL-10 in human serum, which contributes to inhibition of airway inflammation in asthmatic children (Stelmach et al. 2002b). In vitro investigations showed that montelukast increased IL-10 concentration in supernatants from sensitizing allergen-stimulated peripheral blood mononuclear cell culture obtained from children with asthma (Stelmach et al. 2005). However, the effect of montelukast on the level of IL-10 in broncho-alveolar lavage fluid (BALF), which is a key factor related to asthmatic airway inflammation, has not been reported.

On the basis of previous results described above, we came up with the hypothesis that montelukast may elevate the level of IL-10 in BALF and inhibit the activation of NF- κ B, which may be montelukast's new anti-inflammatory mechanisms in asthmatic airway. The present study was designed to validate this hypothesis.

Material and methods

Drugs and reagents

Ovalbumin and poly-L-lysine solution were purchased from Sigma (St. Louis, Mo.). Montelukast was provided by Merck (Rahway, N.J.). IL-10 ELISA kit was purchased from Shanghai Senxiong Technological Company (Shanghai, China). Anti-NF- κ B/p65 antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.).

Animals, sensitization, and study protocol

Healthy Hartly guinea pigs (♂, 300 $\pm$ 50 g) were provided by Experimental Animal Center of Nanjing Medical University. All experimental protocols were approved by our institutional animal care committee and were performed in accordance with the principles and guidelines of the China Council on Animal Care. Forty animals were randomly divided into 5 groups (8 in each group): normal control, model, and montelukast groups (low dose 0.3 mg/kg body mass, medium dose 0.9 mg/kg body mass, high dose 3 mg/kg body mass). Active sensitization was performed by subcuta-

neous injection of 1 mL saline containing 1 mg of chicken egg ovalbumin (OVA) and 3.5 mg of aluminum hydroxide gel. Guinea pigs in normal control were treated with 1 mL of saline only containing 3.5 mg aluminum hydroxide gel instead. In addition, 0.5 mL of *Bordetella pertussis* vaccine containing 2×10^{10} heat-killed organisms was administered intraperitoneally as an adjuvant (Allakhverdi et al. 2000). Two weeks after sensitization, the guinea pigs were challenged with an aerosol of 1% OVA. Guinea pigs in montelukast groups were treated (i.g. intragastric administration) with different doses of montelukast, respectively, 2 h daily before allergen challenge. Guinea pigs in normal control and model group were treated with normal saline. The administration and challenge were performed once a day for a week.

Histopathological observation

Guinea pigs were anesthetized by intraperitoneal injection with sodium pentobarbital (30 mg/kg body mass) 24 h after the last exposure to OVA aerosol. The lower lobes of the right lungs were removed, fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin. Five- μ m-thick sections were serially cut for hematoxylin and eosin (HE) and immunohistochemical staining.

Immunohistochemistry staining for NF- κ B

The slides were removed from the paraffin and were incubated with 3% (v/v) hydrogen peroxide for 10 min at room temperature. After wash and incubation with normal goat blocking serum for 20 min, polyclonal rabbit anti-p65 antibodies (1:100 dilution) were added. The slides were incubated overnight at 4 °C, rinsed 3 times with PBS, and then incubated with a peroxidase-labeled goat anti-rabbit immunoglobulin G (IgG) for 30 min at 37 °C, and then washed again with PBS followed by DAB stain. The sections were counterstained with hematoxylin, dehydrated, and observed under light microscope. The percent of nuclear NF- κ B positive cells to total airway epithelial cells was computed through a RY-2000 computerized image analysis system attached to the microscope (Hart et al. 2000). Five high power fields per slide were observed.

Bronchoalveolar lavage

Bronchoalveolar lavage (Duan et al. 2003; Li et al. 1999) was performed 24 h after the last OVA challenge. Guinea pigs were anesthetized by an intraperitoneal injection of pentobarbital sodium (30 mg/kg body mass). Tracheotomy was performed, and a cannula was inserted into the trachea. Phosphate-buffered saline was instilled into the lungs, and BALF was collected. The recovered BALF were pooled and centrifuged, generating a BALF cell pellet and a cell-free supernatant. Cell-free lavage fluid was frozen and stored at -70 °C until use.

ELISA for IL-10

IL-10 level in BALF was detected by enzyme-linked immunosorbent assay (ELISA) according to the protocols of the kit. Briefly, cell-free bronchoalveolar lavage fluid was added to each well of a monoclonal rabbit anti-rat IL-10 antibody coated micro titre plates (ELISA plates) for 12 h at 4 °C. Unbound material was washed off and a biotiny-

lated monoclonal rabbit anti-rat IL-10 antibody was used at 2 µg/mL for 45 min. Bound antibody was detected by addition of avidin-peroxidase for 30 min followed by incubation of the ABTS substrate solution. Absorbance at 405 nm was measured 20 min after addition of substrate. A standard curve was constructed using various dilutions of IL-10 standard preparation in the kit. The amounts of IL-10 in the BALF were determined by extrapolation of absorbance to the standard curve. The intra-assay and inter-assay variability for the determination was <5%.

Eosinophils purification and count

Eosinophils were isolated from BALF and anticoagulated blood of asthmatic guinea pigs after removal of mononuclear cells by Percoll (Pharmacia Co., Sweden) density-gradient centrifugation (Gartner 1980; Jawien et al. 2004). The depletion of neutrophils by immunomagnetic negative selection as previously described (Masu et al. 2002) was performed. The purity of eosinophils as determined by light microscopic examination of cytospin preparations stained with Diff-Quik (International Chemical Ltd., Osaka, Japan) was higher than 99%. The viability assessed by trypan blue dye exclusion was higher than 99%. The purified eosinophils were counted under the light microscope.

Statistical analysis

All data are expressed as mean $\pm$ SD. The significance for the difference between the groups was analyzed with using one-way ANOVA (SPSS 10.0). Differences were considered significant when $p < 0.05$. The correlation between the activation of NF- κ B and the level of IL-10 was analyzed using Pearson analysis method.

Results

Effect of montelukast on histopathological change in lung tissues

As demonstrated in Fig. 1A, guinea pigs in normal control showed normal bronchial and alveolar architecture. Marked lung histopathologic abnormalities were observed in model group, characterized by bronchostenosis, epithelium hyperplasia, and infiltration of inflammatory cells in bronchial and alveolar regions (Fig. 1B). After treatment with montelukast, lung tissues appeared relatively better, with a prominent decrease in inflammation response (Fig. 1C).

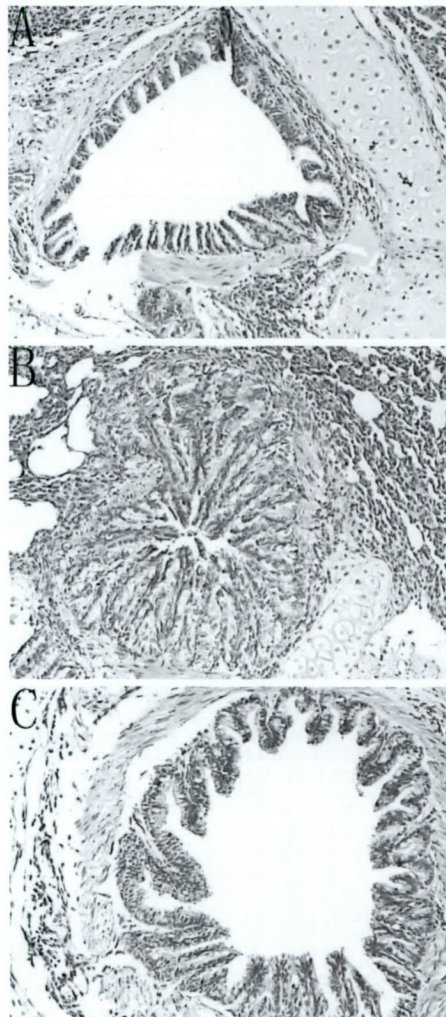
Effect of montelukast on the number of eosinophils

The number of eosinophils in blood and BALF of asthma model guinea pigs increased. There was significant difference between model group and normal control ($n = 8$, $p < 0.01$). Compared with model group, montelukast at 0.9 and 3.0 mg/kg decreases the number of eosinophils in asthmatic guinea pigs significantly ($n = 8$, $p < 0.05$ and $p < 0.01$, respectively; Table 1).

Effect of montelukast on the level of IL-10 in BALF

Compared with normal control, the level of IL-10 in BALF of model group was prominently lower (8.3 ± 2.1 vs. 13.9 ± 2.1 pg/mL, $n = 8$, $p < 0.01$). In contrast, there was a significant increase in level of IL-10 after treatment with

Fig. 1. Effect of montelukast on histopathological changes in lung tissues of asthmatic guinea pigs induced by 1% chicken egg ovalbumin challenge. (A) Normal control; (B) asthma model; and (C) high dose of montelukast (3.0 mg/kg). Pulmonary inflammation was observed by hematoxylin and eosin staining (100 $\times$).



montelukast at medium and high doses compared with model group (12.7 ± 2.6 , $p < 0.01$; 13.4 ± 3.2 , $p < 0.01$; vs. 8.3 ± 2.1 pg/mL, $n = 8$, respectively; Fig. 2).

Table 1. Effect of montelukast (0.3, 0.9, 3.0 mg/kg given i.g. 2 h before chicken egg ovalbumin) on the number of eosinophils in blood and broncho-alveolar lavage fluid of asthmatic guinea pigs induced by OVA challenge.

Group	Dose (mg/kg)	No. of eosinophils ($\times 10^6/\text{mL}$)	
		Blood	Broncho-alveolar lavage fluid
Normal control		1.76 $\pm$ 0.65	1.25 $\pm$ 0.32
Asthma model		11.94 $\pm$ 2.55*	10.89 $\pm$ 2.64*
Montelukast	0.3	10.48 $\pm$ 2.11	9.36 $\pm$ 1.88
	0.9	7.38 $\pm$ 1.12†	6.95 $\pm$ 1.03†
	3.0	2.81 $\pm$ 0.69‡	2.34 $\pm$ 0.76‡

Note: Data are expressed as mean $\pm$ SD, $n = 8$, * $p < 0.01$ vs. normal control; † $p < 0.05$ and ‡ $p < 0.01$ vs. asthma model.

Fig. 2. Effect of montelukast (0.3, 0.9, and 3.0 mg/kg given i.g. 2 h before chicken egg ovalbumin (OVA)) on the level of IL-10 in broncho-alveolar lavage fluid of asthmatic guinea pigs induced by 1% OVA challenge. Data are expressed as mean $\pm$ SD, $n = 8$, ** $p < 0.01$ vs. normal control; † $p < 0.01$ vs. asthma model.

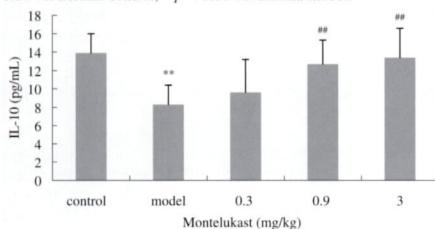
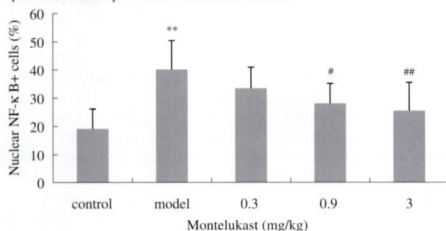


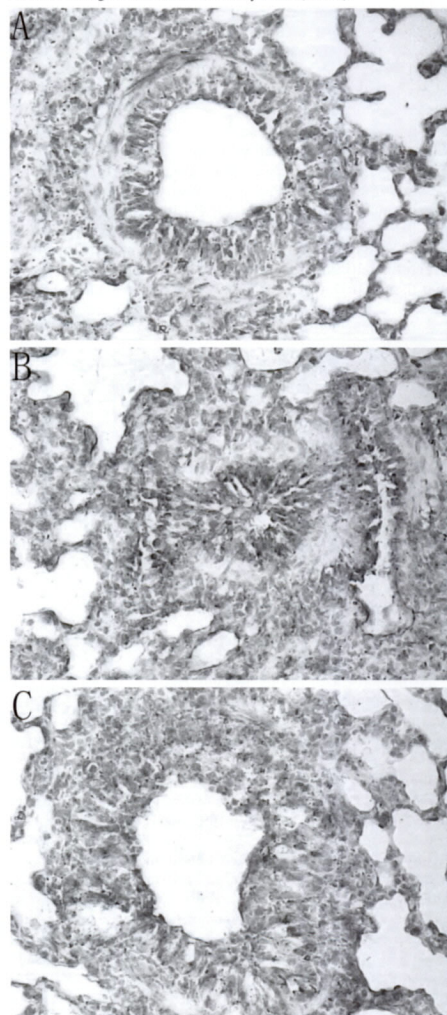
Fig. 3. Effect of montelukast (0.3, 0.9, and 3.0 mg/kg given i.g. 2 h before chicken egg ovalbumin (OVA)) on activation of NF- κ B in lung tissues of asthmatic guinea pigs induced by 1% OVA challenge. The degree of NF- κ B activation was indicated as percentage of nuclear NF- κ B-positive cells to total airway epithelial cells. Data are expressed as mean $\pm$ SD, $n = 8$, ** $p < 0.01$ vs. normal control; † $p < 0.05$ and ‡ $p < 0.01$ vs. asthma model.



Effect of montelukast on activation of NF- κ B in lung tissues

The majority of nuclear positive staining representing the activated form of NF- κ B was observed in the epithelium. In

Fig. 4. Effect of montelukast on the activation of NF- κ B in lung tissues of asthmatic guinea pigs. (A) Normal control; (B) asthma model; and (C) high dose of montelukast (3.0 mg/kg). Tissues were observed using immunohistochemistry stain (200 $\times$).



model group, about 40% of epithelial cells expressed nuclear positive staining, as compared with about 19% in normal control ($n = 8$, $p < 0.01$). The percent of cells showing nuclear positive staining decreased significantly after treat-

ment with montelukast at medium and high doses compared with model group ($28.13\% \pm 7.04\%$, $p < 0.05$; $25.5\% \pm 10.03\%$, $p < 0.01$; vs. $40.13\% \pm 10.32\%$, $n = 8$, respectively; Figs. 3 and 4).

Correlation between the level of IL-10 and activation of NF- κ B

As described above, the level of IL-10 in BALF was the lowest whereas the percent of cells showing nuclear NF- κ B positive staining was the highest in asthma model group. On the other hand, the level of IL-10 in BALF was the highest whereas the percentage of cells showing nuclear NF- κ B positive staining was the lowest in normal control group. In groups treated with montelukast, the level of IL-10 increased and the activation of NF- κ B was inhibited in a dose-dependent manner. The Pearson correlation analysis showed a significantly negative correlation between the level of IL-10 and the activation of NF- κ B was found in lung tissues ($r = -0.488$, $p < 0.01$). In the present study, the correlation between the number of eosinophils and the level of IL-10 or the activation of NF- κ B was not significant.

Discussion

In the present study, we report that montelukast, a potent, specific leukotriene receptor antagonist, prevents the decrease of IL-10 and inhibits the activation of NF- κ B, thereby reducing the severity of inflammation in lung tissues of asthmatic guinea pigs.

Asthma is a chronic lung disease exhibiting airway obstruction, hyperresponsiveness, and inflammation, characterized by the infiltration of many inflammatory cells. It has been demonstrated that montelukast exerts potent anti-inflammation effects on airway of asthmatic patients and animal model. The symptoms of asthmatic patients are alleviated by montelukast (Knorr et al. 1998; Reiss et al. 1996; Reiss et al. 1998). Our results that montelukast markedly reduced the severity of airway inflammation and the number of eosinophils in blood and BALF of asthmatic guinea pigs were consistent with previous reports (Zhang et al. 2004). The precise mechanisms of chronic airway inflammation of asthma are not completely known but are considered to be related to increased various pro-inflammatory mediators and decreased anti-inflammatory cytokines (Barnes 1994).

IL-10 is defined as a cytokine synthesis inhibitory factor. IL-10 was initially identified in mice, where it is a Th2 lymphocyte product that inhibits both Th1 proliferation and production of interferon- γ and IL-2 (Bellinghausen et al. 2001; Matsumoto et al. 2005). Subsequent studies have extended these activities to humans, showing that IL-10 is also a product of Th1 lymphocytes and downregulates IL-4 and IL-5 expression by Th2 lymphocytes. IL-10 is also produced by cytotoxic T cells, B lymphocytes, and mast cells. In contrast to mice, in human monocytes are the major source of IL-10. In addition to its effect on Th1 and Th2 cytokines, IL-10 inhibits the production of IL-1, IL-6, IL-8, IL-12, tumor necrosis factor- α by mononuclear phagocytes and interferon- γ , tumor necrosis factor- α by natural killer cells. Therefore, IL-10 is an important immunoregulatory and anti-inflammatory cytokine. It has been shown that the level of IL-10 is reduced in BALF of asthmatic patients. Increas-

ing the level of IL-10 contributes to the relief of asthmatic airway inflammation (Ceyhan et al. 2004; Lyon et al. 2004; Stelmach et al. 2002a). Our result showed that montelukast prevents the fall of IL-10 in BALF of asthmatic guinea pigs, which may be a pathway for montelukast to exert anti-airway-inflammation effect.

Gene transcription of many asthma-related inflammatory mediators and cytokines is considered to be related to NF- κ B. NF- κ B is an important nuclear transcription factor. NF- κ B heterodimer consists of p50 and p65 (Rel A) subunits. It plays a pivotal role in immune and inflammatory responses through the regulation of the expression of several proteins, including pro-inflammatory cytokines, chemokines, and adhesion molecules. Uncontrolled activation of the NF- κ B pathway is involved in the pathogenesis of several chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease (Yamamoto and Gaynor 2004). Several lines of evidence suggest that NF- κ B also plays a central role in the pathogenesis of asthma (Wright and Christman 2003). Activated NF- κ B has been identified in the airways of asthmatic patients, and agents such as allergens, ozone, and viral infections, which are associated with exacerbation of asthma, stimulate activation of NF- κ B. In addition, the major effective treatment for asthma, corticosteroids, is a potent blocker of NF- κ B activation. Finally, mice lacking the NF- κ B p50 or c-Rel subunits develop less airway inflammation on antigen challenge (Donovan et al. 1999; Yang et al. 1998). It has been demonstrated that the activation of NF- κ B occurs predominantly in the bronchiolar epithelium in the lung. In its inactive state, the NF- κ B dimer is present in the cytosol, where it is bound to an inhibitory protein, I- κ B. Activation of NF- κ B by several stimuli, including cytokines and microorganisms, induces the release and degradation of the inhibitory protein I- κ B from the dimeric complex (Bauerle and Baltimore 1988; Bauerle and Henkel 1994), followed by translocation of NF- κ B to the nucleus (Grilli et al. 1993; Muller et al. 1993). In the nucleus, NF- κ B is bound to corresponding sites to regulate many of the cytokine and adhesion molecule genes expressed in asthma. Because of the potential role of NF- κ B in airway inflammation, it has been proposed that inhibitors of NF- κ B activation may have therapeutic potential in allergic lung inflammation (Barnes 2003; Popescu 2003). Recent study indicates that montelukast inhibited NF- κ B activation in THP-1 cells (a human monocytic leukemia cell line) in a dose-related manner (Maeba et al. 2005). Furthermore, 10^{-5} mol/L montelukast significantly inhibited lipopolysaccharide-induced IL-6, tumor necrosis factor- α , and monocyte chemoattractant protein 1 production in the peripheral blood mononuclear cells. High doses of montelukast could modulate the production of proinflammatory cytokines and chemokines through the inhibition of NF- κ B activation (Maeba et al. 2005). Our *in vivo* result that montelukast at medium and high doses could significantly inhibit the nucleus expression of NF- κ B in lung of asthmatic guinea pigs was consistent with the previous study. It highly implies that montelukast may exert anti-inflammation effect by inhibiting activation of NF- κ B in asthmatic airway.

We also evaluated the correlation between the level of IL-10 and the activation of NF- κ B in lung tissues. The result

showed that the inhibiting of NF- κ B activation was accompanied by the increasing of IL-10 level. There was a significantly negative correlation between them. These data are consistent with the theory of a negative correlation between IL-10 and NF- κ B (Saadane et al. 2005).

In conclusion, our study demonstrated that montelukast exerted beneficial effects on asthmatic airway inflammation and discovered new anti-inflammation mechanisms of montelukast. Preventing the decrease of IL-10 and inhibiting the activation of NF- κ B may be important mechanisms for montelukast exerting anti-inflammation effect in inflammatory airway of asthmatic guinea pigs.

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