Cysteinyl leukotriene receptor antagonist regulates vascular permeability by reducing vascular endothelial growth factor expression

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Background: Inflammation of the asthmatic airway is usually accompanied by increased vascular permeability and plasma exudation. Cysteinyl leukotrienes (cysLTs) potently elicit increased vascular permeability in airways, leading to airway edema. Vascular endothelial growth factor (VEGF) is 1 of the most potent proangiogenic cytokines and also increases vascular permeability so that plasma proteins can leak into the extravascular space. However, the mechanisms by which cysLTs induce increased vascular permeability are not clearly understood.

Objective: An aim of the current study was to determine the role of the cysLTs, more specifically in the increase of vascular permeability.

Methods: We used a BALB/c mouse model of allergic asthma to examine effects of cysLT receptor antagonists on bronchial inflammation and airway hyperresponsiveness, more specifically on the increase of vascular permeability.

Results: These mice develop the following typical pathophysiological features of asthma in the lungs: increased numbers of inflammatory cells of the airways, airway hyperresponsiveness, increased vascular permeability, and increased levels of VEGF. Administration of cysLT receptor antagonists markedly reduced plasma extravasation and VEGF levels in allergen-induced asthmatic lungs.

Conclusion: These results indicate that cysLT receptor antagonists modulate vascular permeability by reducing VEGF expression and suggest that cysLT receptor may regulate the VEGF expression. (J Allergy Clin Immunol 2004;114:1093-9.)

Key words: Cysteinyl leukotrienes, vascular endothelial growth factor, asthma, vascular permeability

Asthma is a chronic airway inflammatory disease characterized by an infiltration of inflammatory cells, including eosinophils, mast cells, and T lymphocytes.1 Leukotrienes (LTs), lipid mediators generated from arachidonic acid by the action of 5-lipoxygenase, play important roles in the pathogenesis of allergic airway inflammation.2 LTC4, LTD4, and LTE4, known as cysteinyl leukotrienes (cysLTs), are both direct bronchoconstrictors and proinflammatory substances and are involved in recruitment of inflammatory cells, airway smooth muscle contraction, vascular leakage, increased mucus secretion, and decreased mucociliary clearance.3 cysLTs are shown to cause plasma extravasation in vivo and in vitro studies.4-10 Inflammation of the asthmatic airway is usually accompanied by increased vascular permeability and plasma exudation. However, the mechanisms by which cysLTs induce increased vascular permeability are not clearly understood.

Vascular endothelial growth factor (VEGF) is an endothelial cell–specific mitogenic peptide and plays a key role in vasculogenesis and angiogenesis.11 VEGF also increases vascular permeability so that plasma proteins can leak into the extravascular space, which leads to edema and profound alterations in the extracellular matrix. Recently, we have demonstrated that VEGF is 1 of the major determinants of asthma and that the inhibition of VEGF receptor may be a good therapeutic strategy.12,13

In the current study, we used a murine model of asthma to determine the role of the cysLTs, specifically in the increase of vascular permeability. We found evidence that cysLT receptor antagonists inhibit increased vascular permeability, airway inflammation, and airway hyperresponsiveness. In addition, cysLT receptor antagonists reduce VEGF expression.

METHODS

Animals and experimental protocol

Female BALB/c mice, 8 to 10 weeks old and free of murine-specific pathogens, were obtained from the Korean Research Institute...
of Chemistry Technology (Daejon, Korea). All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School. Standard guidelines for laboratory animal care were followed. Mice were sensitized and challenged, as previously described (see Fig E1 in the Journal’s Online Repository at www.mosby.com/jaci).15

Bronchoalveolar lavage (BAL) was performed at 72 hours after the last challenge. After killing by using sodium pentobarbitone (pentobarbital sodium, 100 mg/kg body weight), prewarmed 0.9% NaCl solution was slowly infused into the lungs and withdrawn. Total cell numbers were counted with a hemocytometer. Smears of BAL cells prepared with a Cytospin II (Shandon, Runcorn, United Kingdom) were stained with Diff-Quik solution (Dade Diagnostics of Puerto Rico, Aguada) for differential cell counting. Two independent, blind investigators counted the cells by using a microscope. Approximately 400 cells were counted in each of 4 random locations.

Administration of montelukast, pranlukast, or VEGF receptor inhibitor

Montelukast sodium (L-706, 631; 5 mg/kg body weight/d; Merck & Co, Rahway, NJ) dissolved in 1% methyl cellulose or pranlukast (25 mg/kg body weight/d; Ono Pharmaceutical Co Ltd, Osaka, Japan) dissolved in distilled water was administered by oral gavage 3 times at 24-hour intervals beginning 1 hour after the last airway challenge with ovalbumin (OVA), as previously described (see Fig E1 in the Online Repository at www.mosby.com/jaci). An inhibitor of VEGF receptor tyrosine kinase, SU5614 (Flk-1; 25 mg/kg body weight/d; Merck & Co, Rahway, NJ) dissolved in 1% methyl cellulose or pranlukast (25 mg/kg body weight/d; Ono Pharmaceutical Co Ltd, Osaka, Japan) dissolved in distilled water was administered by oral gavage 7 times at 24-hour intervals on days 19 to 25, beginning 2 days before the first challenge (see Fig E1 in the Online Repository at www.mosby.com/jaci). An inhibitor of VEGF receptor tyrosine kinase, SU5614 (Flk-1; IC50 = 1.2 ìmol/L, 5-chloro-3-[(3,5-dimethylpyrrol-2-yl)methylene]-2-indolinone; Calbiochem, San Diego, Calif), was used to inhibit VEGF activity. SU5614 (2.5 mg/kg body weight/d) was dissolved in dimethyl sulfoxide and administered intraperitoneally 3 times at 24-hour intervals beginning 1 hour after the last airway challenge with ovalbumin (OVA), as previously described (see Fig E1 in the Online Repository at www.mosby.com/jaci).15

Measurement of plasma exudation

To assess lung permeability, Evans blue dye (EBD) was dissolved in 0.9% saline at a final concentration of 5 mg/mL. Animals were weighed and injected with 20 mg/kg EBD in the tail vein. After 30 minutes, the animals were killed, and their chests were opened. Normal saline containing 5 mmol/L ethylene diamine tetra-acetic acid was perfused through the aorta until all venous fluid returning to the opened right atrium was clear. The lungs were removed and weighed wet. EBD was extracted in 2 mL formamide kept in a water bath at 60°C for 3 hours, and the absorption of light at 620 nm was measured in a spectrophotometer (Spectra Max Plus Microplate Spectrophotometer, Sunnyvale, Calif). The dye extracted was quantified by interpolation against a standard curve of dye concentration of 0.01 to 10 µg/mL and is expressed as nanograms of dye per milligram of wet lung.

Isolation of BAL fluid eosinophils

Eosinophils were purified as previously described.15,16

Measurements of VEGF in BAL fluids

Levels of VEGF were quantified by an enzyme immunoassay according to the manufacturer’s protocol (R & D Systems, Minneapolis, Minn).

Histology, immunohistochemistry, and immunocytochemistry

Four-micrometer sections of fixed, embedded tissues were cut, placed on glass slides, deparaffinized, and stained sequentially with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, Mich). For immunohistochemistry and immunocytochemistry of VEGF, the deparaffinized 4 µm sections, the cytocentrifuge preparations of BAL cells, or BAL eosinophils were incubated sequentially in accordance with the RTU Vectastain Universal Quick kit instruction from Vector Laboratories (Burlingame, Calif). The slides were probed with an affinity-purified rabbit polyclonal VEGF IgG (Santa Cruz Biotechnology, Santa Cruz, Calif) overnight at 4°C.

Cytosolic and nuclear protein extractions for analysis of nuclear factor κB p65

Lungs were removed and homogenized in 2 volumes of buffer A (50 mmol/L Tris-HCl, pH 7.5, 1 mmol/L ethylene diamine tetraacetic acid, 10% glycerol, 0.5 mmol/L dithiothreitol, 5 mmol/L MgCl2, and 1 mmol/L phenylmethylsulfonyl fluoride) containing protease inhibitor cocktails. These homogenates were centrifuged at 1000g for 15 minutes at 4°C. The supernatant fraction was incubated on ice for 10 minutes and centrifuged at 100,000g for 1 hour at 4°C to obtain cytosolic protein extracts. The pellets were washed twice in buffer A and resuspended in buffer B (1.3 mol/L sucrose, 1.0 mmol/L MgCl2, and 10 mmol/L potassium phosphate buffer, pH 6.8) and pelleted at 100,000g for 15 minutes. The pellets were suspended in buffer B with a final sucrose concentration of 2.2 mol/L and centrifuged at 100,000g for 1 hour. The resulting nuclear pellets were washed once with a solution containing 0.25 mol/L sucrose, 0.5 mmol/L MgCl2, and 20 mmol/L Tris-HCl, pH 7.2, and centrifuged at 100,000g for 10 minutes. The pellets were solubilized with a solution containing 50 mmol/L Tris-HCl (pH 7.2), 0.3 mol/L sucrose, 150 mmol/L NaCl, 2 mmol/L ethylene diamine tetraacetic acid, 20% glycerol, 2% Triton X-100, 2 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktails. The mixture was kept on ice for 1 hour with gentle stirring and centrifuged at 12,000g for 30 minutes. The resulting supernatant was used as soluble nuclear proteins.

Western blot analysis

Western blot analysis was performed as described previously.13,15 The blots were incubated with an antimiouse VEGF antibody (Santa Cruz Biotechnology) or anti–nuclear factor κB (NF-κB) p65 (Upstate Biotech, Lake Placid, NY) overnight at 4°C.

Determination of airway responsiveness to methacholine

Airway responsiveness was measured in mice 3 days after the methacholine challenge in an unrestrained conscious state, as previously described.13,15

Measurement of LTC4

Levels of LTC4 were quantified in the supernatants of BAL fluids by enzyme immunoassay according to the manufacturer’s protocol (Cayman Chemical Co, Ann Arbor, Mich). Sensitivity for the assay was 10 pg/mL.

Measurement of cytokines

Levels of IL-4 and IL-5 were quantified in the supernatants of BAL fluids by enzyme immunoassays according to the manufacturer’s protocol (Endogen, Woburn, Mass).

Measurement of eosinophil cationic protein

Eosinophil cationic protein (ECP) was measured in the supernatants of BAL fluids. Levels of ECP were quantified by fluoroenzymeimmunoassay according to the manufacturer’s protocol (UniCAP ECP; Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden).
Statistical analysis

Data are expressed as means ± SEMs. Statistical comparisons were performed by using 1-way ANOVA followed by the Fisher test. Significant differences between groups were determined by using the unpaired Student t test. Statistical significance was set at P < .05.

RESULTS

Effect of montelukast or pranlukast on leukotriene C levels in BAL fluids

Levels of leukotriene C in BAL fluids were increased significantly at 72 hours after OVA inhalation compared with levels after saline inhalation (see Fig E2 in the Online Repository at www.mosby.com/jaci). The increased leukotriene C levels at 72 hours after OVA inhalation were significantly reduced by the administration of montelukast or pranlukast.

Effect of montelukast, pranlukast, or SU5614 on cellular changes in BAL fluids

Numbers of total cells and eosinophils in BAL fluids were increased significantly at 72 hours after OVA inhalation compared with numbers after saline inhalation (Fig 1). The increased numbers of eosinophils at 3 days after OVA inhalation were significantly reduced by the administration of montelukast or pranlukast, or SU5614.

Effect of montelukast or pranlukast on pathological changes of OVA-induced asthma

Histologic analyses revealed typical pathologic features of asthma in the OVA-exposed mice. Numerous inflammatory cells, including eosinophils, infiltrated around the bronchioles; the airway epithelium was thickened; and mucus and debris had accumulated in the lumen of bronchioles (Fig 2, B). Mice treated with montelukast (Fig 2, C) or pranlukast (Fig 2, D) showed marked reductions in the thickening of airway epithelium, in the infiltration of inflammatory cells in the peribronchiolar region, in the number of inflammatory cells, and in the amount of debris in the airway lumen. These results indicate that montelukast and pranlukast inhibit antigen-induced inflammation in the lungs, including the influx of eosinophils.

Effect of montelukast or pranlukast on levels of IL-4, IL-5, and ECP in BAL fluids

Levels of IL-4 and IL-5 in BAL fluids were increased significantly at 72 hours after OVA inhalation compared with levels after saline inhalation (see Fig E3 in the Online Repository at www.mosby.com/jaci). The increased IL-4 and IL-5 levels at 72 hours after OVA inhalation were significantly reduced by the administration of montelukast or pranlukast. In addition, the increased ECP levels in the BAL fluids at 72 hours after OVA inhalation were significantly decreased by the administration of montelukast or pranlukast (see Fig E3 in the Online Repository at www.mosby.com/jaci). These results suggest that montelukast and pranlukast inhibit the antigen-induced release of soluble mediators of inflammation into the lungs.

cysLT receptor antagonists and VEGF receptor inhibitor reduced OVA-induced airway hyperresponsiveness

Airway responsiveness was assessed as a percent increase of enhanced pause (Penh) in response to increasing doses of methacholine. In OVA-sensitized and OVA-challenged mice, the dose-response curve of percent Penh shifted to the left compared with that of control mice (Fig 3). In addition, the percent Penh produced by methacholine administration (at doses 2.5-50 mg/mL) increased significantly in the OVA-sensitized and OVA-challenged mice compared with the controls. OVA-sensitized and OVA-challenged mice treated with montelukast, pranlukast, or SU5614 showed a dose-response...
Mechanisms of asthma and allergic inflammation

curve of percent Penh that shifted to the right compared with that of untreated mice. These results indicate that montelukast, pranlukast, or SU5614 treatment reduces OVA-induced airway hyperresponsiveness.

**cysLT receptor antagonists and VEGF receptor inhibitor reduced plasma extravasation in OVA-sensitized and OVA-challenged mice**

The EBD assay revealed that plasma extravasation was significantly increased at 72 hours after the last challenge (Fig 4). The increase in plasma extravasation at 72 hours after OVA inhalation was significantly reduced by the administration of the cysLT receptor antagonists or VEGF receptor inhibitor.

**cysLT receptor antagonists and VEGF receptor inhibitor decreased VEGF levels in BAL fluids of OVA-sensitized and OVA-challenged mice**

Administration of montelukast, pranlukast, or SU5614 dramatically reduced the increased levels of VEGF in BAL fluids at 72 hours after the last challenge (Fig 5, A). Consistent with the results obtained from the enzyme immunoassay, Western blot analysis revealed that montelukast, pranlukast, or SU5614 reduced the increased levels of VEGF in the BAL fluid at 72 hours after OVA inhalation (Fig 5, B).

**Localization of immunoreactive VEGF in lung tissues and BAL fluids of OVA-induced asthma**

Immunohistochemical analyses showed the localization of immunoreactive VEGF in inflammatory cells around the bronchioles of mice with OVA-induced asthma (Fig 6, B). In control mice and in OVA-sensitized and OVA-challenged mice treated with montelukast or pranlukast, almost no VEGF positive cells were detected (Fig 6, A, C, and D). Immunocytologic analysis of BAL fluids showed the localization of immunoreactive VEGF in the precipitated cells from the OVA-sensitized and OVA-challenged mice (Fig 6, F). However, immunoreactive VEGF was significantly reduced in BAL cells from control mice and from OVA-sensitized and OVA-challenged mice treated with montelukast or pranlukast.
In addition, the expression of VEGF in BAL fluid eosinophils isolated by Percoll gradients was also examined by means of immunocytochemistry. Localization of immunoreactive VEGF was observed in BAL fluid eosinophils from the OVA-sensitized and OVA-challenged mice (Fig 6, I). However, immunoreactive VEGF was markedly reduced in BAL fluid eosinophils from OVA-sensitized and OVA-challenged mice treated with montelukast or pranlukast (Fig 6, J and K).

Effect of montelukast or pranlukast on NF-κB p65 protein levels in lung tissues of OVA-sensitized and OVA-challenged mice

Western blot analysis revealed that levels of NF-κB p65 protein in nuclear protein extracts from lung tissues were increased at 72 hours after OVA inhalation compared with the levels in the control mice (Fig 7). The increased NF-κB p65 levels in nuclear protein extracts from lung tissues at 72 hours after OVA inhalation were decreased by the administration of montelukast or pranlukast. In contrast, levels of NF-κB p65 protein in cytosolic protein extracts from lung tissues were decreased at 72 hours after OVA inhalation compared with the levels in the control mice (Fig 7). The decreased NF-κB p65 levels in cytosolic protein extracts from lung tissues at 72 hours after OVA inhalation were increased by the administration of montelukast or pranlukast. These results indicate that montelukast or pranlukast inhibits NF-κB activity by preventing translocation of this transcription factor into nucleus.

DISCUSSION

Bronchial asthma is characterized by inflammation of the airways, which is usually accompanied by increased vascular permeability, resulting in plasma exudation. It is well known that cysLTs play critical roles in airway inflammation. Although cysLTs are shown to cause plasma extravasation in in vivo and in vitro studies, direct evidence for the involvement of cysLTs in pathogenesis of asthma, including the vascular permeability, is lacking. In this study, we have examined effects of cysLTs on the increase of vascular permeability by using cysLT receptor antagonists, montelukast and pranlukast. The results show that plasma extravasation caused by increased vascular permeability is elevated after inducing
asthma and that administration of either montelukast or pranlukast significantly reduces the increased plasma extravasation at 72 hours after OVA inhalation. Moreover, administration of the antagonists decreases eosinophilic inflammation and airway hyperresponsiveness.

Evidence for the increased vascular permeability in our murine asthma model is that amounts of plasma extravasation and levels of VEGF are greatly enhanced at 72 hours after the last OVA challenge. Increased vascular permeability causes secretion of intravascular components, including plasma extravasation. Although the pathogenesis of asthma induced by plasma extravasation is not clearly defined, plasma protein leakage has been implicated in the induction of a thickened, engorged, and edematous airway wall, resulting in the airway lumen narrowing. Van de Graaf et al.\(^{18}\) have reported that exudation of plasma proteins into the airways correlates with bronchial hyperreactivity. It is also possible that the plasma exudate may readily pass the inflamed mucosa and reach the airway lumen through leaky epithelium, thus compromising epithelial integrity and reducing ciliary function and mucus clearance.\(^{19,20}\)

Recently, we reported that overproduction of VEGF is associated with increased vascular permeability and plasma exudation in toluene di-isocyanate–induced asthma.\(^ {13}\) Consistent with these observations, in this study we have found that VEGF expression is upregulated in OVA-induced asthma. Interestingly, administration of the cysLT receptor antagonist, montelukast or pranlukast, reduces the increased VEGF expression. These results suggest that cysLT receptor signaling is associated with the regulation of VEGF expression and that treatment of the cysLT receptor antagonists may decrease the vascular permeability by inhibiting upregulation of VEGF expression. It is now well established that VEGF plays a critical role in asthma.\(^ {12,23}\) The major role of VEGF in asthma appears to be the enhancement of vascular permeability, resulting in leakage of plasma proteins into the extravascular space.\(^ {11,13}\) This may cause edema and profound alterations in the extracellular matrix. The mechanism of VEGF-mediated induction of the vascular permeability seems to be the enhanced functional activity of vesicular–vacuolar organelles.\(^ {11,22}\) To distinguish direct effects of cysLT on the asthma phenotype from those of VEGF, VEGF receptor inhibitor, SU5614 was administered. We have found that SU5614 inhibits increased vascular permeability, airway inflammation, and airway hyperresponsiveness. These results suggest that VEGF may affect vascular permeability without affecting leukotriene production.

Previous reports have demonstrated that NF-κB plays a critical role in immune and inflammatory responses, including asthma.\(^ {23-28}\) Activation of NF-κB in allergic airway inflammation has also been observed.\(^ {25,30}\) Determination of NF-κB protein level in nuclear extracts has revealed that this protein level is substantially increased in our current OVA-induced model of asthma, suggesting that NF-κB is activated. The administration of montelukast or pranlukast results in significant reduction of nuclear NF-κB level as well as expression of VEGF. Because it is known that VEGF expression is regulated through NF-κB expression,\(^ {31,32}\) these results strongly suggest that cysLT receptor antagonists may inhibit VEGF expression in asthma by inhibiting activity of NF-κB.

In summary, we have examined the role of the cysLTs in a murine model of allergic asthma, more specifically in the increase of vascular permeability. By using montelukast and pranlukast, specific cysLT receptor antagonists, we have shown the important role for cysLTs in OVA-induced airway hyperresponsiveness and eosinophilic inflammation. By examining the effects of administration of montelukast and pranlukast on plasma exudation and VEGF expression, we conclude that cysLT receptor antagonists modulate vascular permeability by reducing VEGF expression.

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REFERENCES


