

Vascular Endothelial Growth Factor Modulates Matrix Metalloproteinase-9 Expression in Asthma

Kyung Sun Lee*, Kyung Hoon Min*, So Ri Kim, Seoung Ju Park, Hee Sun Park, Gong Yong Jin, and Yong Chul Lee

Department of Internal Medicine, Department of Radiology, Airway Remodeling Laboratory, and Research Center for Allergic Immune Diseases, Chonbuk National University Medical School, Jeonju, Republic of Korea

Rationale: Vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9) are mediators of airway inflammation and remodeling in asthma.

Objectives: This study investigates a potential relationship between VEGF and MMP-9, and the mechanisms by which VEGF signaling regulates MMP-9 expression in asthma.

Methods: We evaluated whether levels of VEGF correlated with levels of MMP-9 in the sputum of asthma patients, and the effect of VEGF receptor inhibitors on MMP-9 expression in murine model of asthma.

Measurements and Main Results: We have found that levels of VEGF and MMP-9 are significantly higher in the sputum of patients with asthma than in healthy control subjects, and a significant correlation is found between the levels of VEGF and MMP-9. This study with the ovalbumin-induced model of asthma revealed the following typical pathophysiologic features of asthma in the lungs: increased numbers of inflammatory cells of the airways, airway hyperresponsiveness, increased vascular permeability, and increased levels of MMP-9 and VEGF. Administration of VEGF receptor inhibitors reduced the pathophysiologic signs of asthma and decreased the increased expression of MMP-9 after ovalbumin inhalation.

Conclusions: These results indicate that there is a close relationship between VEGF and MMP-9 expression and that inhibition of VEGF receptor down-regulates the expression of MMP-9. These findings suggest that VEGF signaling regulates MMP-9 expression and plays a critical role in initiation and maintenance of asthma.

Keywords: airway inflammation; extracellular matrix; permeability; remodeling

Bronchial asthma is a chronic inflammatory disease of the airways that is characterized by exaggerated T-helper 2 (Th2) cell inflammation and airway remodeling. Airway remodeling is due, at least in part, to an excess of extracellular matrix deposition in the airway wall, which leads to subepithelial collagen deposition (1). This airway remodeling has been speculated to be irreversible airway obstruction and one of the factors that makes it difficult to treat patients with asthma (2). The histologic characteristics of chronic inflammation include angiogenesis, increased

connective tissue deposition, and cellular proliferation of myofibroblasts (3). An increase in vessel size, vessel number, and vascular surface area, and the exaggerated expression of vascular endothelial growth factor (VEGF), are documented in the asthmatic airway (4–12). These vascular alterations have been suggested to contribute to the airway obstruction or airway hyperresponsiveness (13–15). VEGF is an endothelial cell-specific mitogenic peptide and plays a key role in vasculogenesis and angiogenesis (16). VEGF also increases vascular permeability so that plasma proteins, including inflammatory mediators and cells, can leak into the extravascular space to allow the migration of inflammatory cells into the airways (14, 17, 18). VEGF is a mediator of vascular and extravascular remodeling and inflammation that enhances antigen sensitization and is crucial in adaptive Th2-cell-mediated inflammation; the inhibition of VEGF receptor may be a good therapeutic strategy (7, 17, 19).

The matrix metalloproteinases (MMPs) are a large family of zinc- and calcium-dependent endopeptidases, with distinct substrate specificities, that can degrade most components of extracellular matrix. The MMPs play an important role in physiologic and pathologic processes, including extracellular matrix turnover, tissue degradation and repair, cell migration, and inflammation. Of the MMP family, MMP-9 is one of the major proteinases that are involved in airway inflammation and bronchial remodeling in asthma (20–22). In addition, MMP-9 induces migration of eosinophils, lymphocytes, and neutrophils across basement membranes (23–25). There are few data on the relationship between VEGF and MMP-9 in asthma, however, and the mechanisms by which VEGF modulates MMP-9 expression have not been clarified.

The present study evaluates whether levels of VEGF correlate with levels of MMP-9 in the sputum of patients with asthma. An additional aim of the present study is to evaluate the effect of VEGF receptor inhibitors on MMP-9 expression in murine model of asthma. Some of the results of these studies have been previously reported in the form of an abstract (26).

METHODS

Subjects

Nineteen asymptomatic patients with stable asthma and 19 patients with acute asthma were recruited from the Chonbuk National University Hospital (Table E1 of the online supplement). Eighteen normal healthy subjects were also selected for this study. This study was approved by the Ethics Committee of Chonbuk National University Medical School and fully informed, written consent was obtained from each subject.

Sputum Collection and Processing

Spontaneous sputum samples were obtained. If spontaneous sputum could not be obtained, induced sputum was obtained. Sputum induction and processing were performed according to the method of Fahy and colleagues (27), with slight modifications.

Measurements of VEGF and MMP-9 in Sputum

Levels of VEGF and MMP-9 were determined by enzyme immunoassays according to the manufacturer's protocol (R&D Systems, Inc.,

(Received in original form October 4, 2005; accepted in final form April 25, 2006)

* These authors contributed equally to this work.

Supported by a Korea Research Foundation grant funded by Korea Government (MOEHRD, Basic Research Promotion Fund; KRF-2005-201-E00014) and a grant from the National Research Laboratory Program of the Korea Science and Engineering Foundation, Republic of Korea.

Correspondence and requests for reprints should be addressed to Yong Chul Lee, M.D., Ph.D., Department of Internal Medicine, Chonbuk National University Medical School, San 2-20 Geumam-dong, Deokjin-gu, Jeonju, Jeonbuk 561-180, Republic of Korea. E-mail: leeyc@chonbuk.ac.kr

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 174, pp 161–170, 2006

Originally Published in Press as DOI: 10.1164/rccm.200510-1558OC on April 27, 2006
Internet address: www.atsjournals.org

Minneapolis, MN). The hydrolytic activity of MMP-9 was measured by gelatin-zymography, as previously described (28). Western blot analysis of VEGF and MMP-9 in sputum was performed using 3 μ g of sputum protein from sputum supernatant.

Animals and Experimental Protocol

Female C57BL/6 mice, 8 to 10 wk of age and free of murine-specific pathogens, were used. All experimental animals used in this study were treated according to guidelines approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School. Mice were sensitized and challenged as previously described (Figure E1) (29).

Administration of VEGF Receptor Inhibitors

Inhibitors of VEGF receptor tyrosine kinase were administered as shown in Figure E1.

Western Blot Analysis

Western blot analysis of Th2 cell cytokine, VEGF, and MMP-9 in lungs of mice was performed as described previously (29).

Measurement of Plasma Exudation

To assess lung permeability, Evans blue dye was used as described previously (17).

Histology, Immunohistochemistry, and Immunocytochemistry

Histologic examination and immunohistochemistry and immunocytochemistry of MMP-9 were performed as described previously (30).

Measurements of VEGF and pro-MMP-9 in Bronchoalveolar Lavage Fluids

Levels of VEGF and pro-MMP-9 were quantified in the supernatants of bronchoalveolar lavage (BAL) fluid by enzyme immunoassays according to the manufacturer's protocol (R&D Systems, Inc.).

Determination of Airway Responsiveness

Airway responsiveness was assessed as a change in airway function after challenge with aerosolized methacholine via airways, as described elsewhere (31).

Densitometric Analyses and Statistics

All immunoreactive and phosphorylation signals were analyzed by densitometric scanning (Gel Doc XR; Bio-Rad Laboratories, Inc., Hercules, CA). Data were expressed as mean $\pm$ SEM. For statistical analysis in patients with asthma and in control subjects, the Kruskal-Wallis analysis of variance test was used to examine significant intergroup differences and, if significant, the Mann-Whitney U test was used for between-group comparisons. Spearman rank correlation was calculated to assess the correlation among data. Comparisons of the time course of VEGF and MMP-9 in the sputum were made through an analysis of variance and Scheffé test. For statistical analysis in mice, one-way analysis of variance followed by the Scheffé test was used. Significant differences between groups were determined using the unpaired Student *t* test. Statistical significance was set at $p < 0.05$.

RESULTS

Characteristics of Subjects and Cell Counts in Their Sputum

Mean ages of patients with stable asthma or acute asthma and of healthy control subjects were similar (Table E1). Initial FEV₁ values of patients with acute asthma ($43 \pm 2.6\%$ of predicted values) were significantly lower than subjects with stable asthma and healthy control subjects ($90.2 \pm 1.4\%$ and $103.8 \pm 1.5\%$ of predicted values, respectively; Table E1). Total cell count was significantly greater in patients with acute asthma (Day 1) compared with patients with stable asthma and healthy control subjects (Table E2). The percentages of eosinophils were higher in patients with stable asthma than in healthy control subjects. The percentages of neutrophils, lymphocytes, and eosinophils were

greater in patients with acute asthma (Day 1) than in patients with stable asthma.

VEGF Levels in Sputum

The levels of VEGF in sputum were significantly increased in patients with stable asthma and even higher in patients with acute asthma compared with healthy control subjects. The levels of VEGF in patients with acute asthma were significantly higher than in patients with stable asthma (Figures 1A and 1B). The time course of VEGF levels in patients with acute asthma before and during treatment is shown in Figures E2A and E2B. The elevated VEGF levels on Day 1 had significantly decreased by Day 3 and even further by Day 5 and Day 30. Consistent with the results obtained from the enzyme immunoassay, Western blot analysis revealed that the levels of VEGF were increased in patients with stable asthma and were even higher in patients with acute asthma compared with healthy control subjects. The elevated VEGF levels in patients with acute asthma on Day 1 had significantly decreased by Day 5 and even further by Day 30. In healthy control subjects and in subjects with stable and acute asthma, the levels of VEGF in sputum were significantly correlated with the number of neutrophils and eosinophils (Figures 2A and 2B).

MMP-9 Levels in Sputum

The levels of MMP-9 in sputum were significantly increased in patients with stable asthma and were even higher in patients with acute asthma compared with healthy control subjects. The levels of MMP-9 in patients with acute asthma were significantly higher than in patients with stable asthma (Figures 1C and 1D). The time course of MMP-9 levels in patients with acute asthma before and during treatment is shown in Figures E2C and E2D. The elevated MMP-9 levels on Day 1 had significantly decreased by Day 3 and even further by Day 5 and Day 30. Consistent with the results obtained from the enzyme immunoassay, Western blot analysis revealed that the levels of MMP-9 were increased in patients with stable asthma and were even higher in patients with acute asthma compared with healthy control subjects. The elevated MMP-9 levels in patients with acute asthma on Day 1 had significantly decreased by Day 5 and even further by Day 30. In addition, zymographic analysis showed the presence of the 92-kD pro-MMP-9 band (Figure 1E). The activities of pro-MMP-9 were increased in patients with stable asthma and were even higher in patients with acute asthma compared with control subjects. In healthy control subjects and in subjects with stable and acute asthma, the levels of MMP-9 in sputum were significantly correlated with the number of neutrophils and eosinophils (Figures 2C and 2D).

Correlation between VEGF and MMP-9 Levels in the Sputum Obtained from Healthy Control Subjects and from Subjects with Stable and Acute Asthma

In the sputum obtained from healthy control subjects and from subjects with stable and acute asthma, the levels of MMP-9 correlated significantly with those of VEGF (Figure 3).

Correlation between the Concentrations of VEGF or MMP-9 in the Sputum and Percent-predicted FEV₁ in Healthy Control Subjects and in Subjects with Stable and Acute Asthma

The levels of VEGF or MMP-9 in sputum obtained from healthy control subjects and from subjects with stable and acute asthma were significantly inversely correlated with the percent-predicted FEV₁ (Figures E3A and E3B).

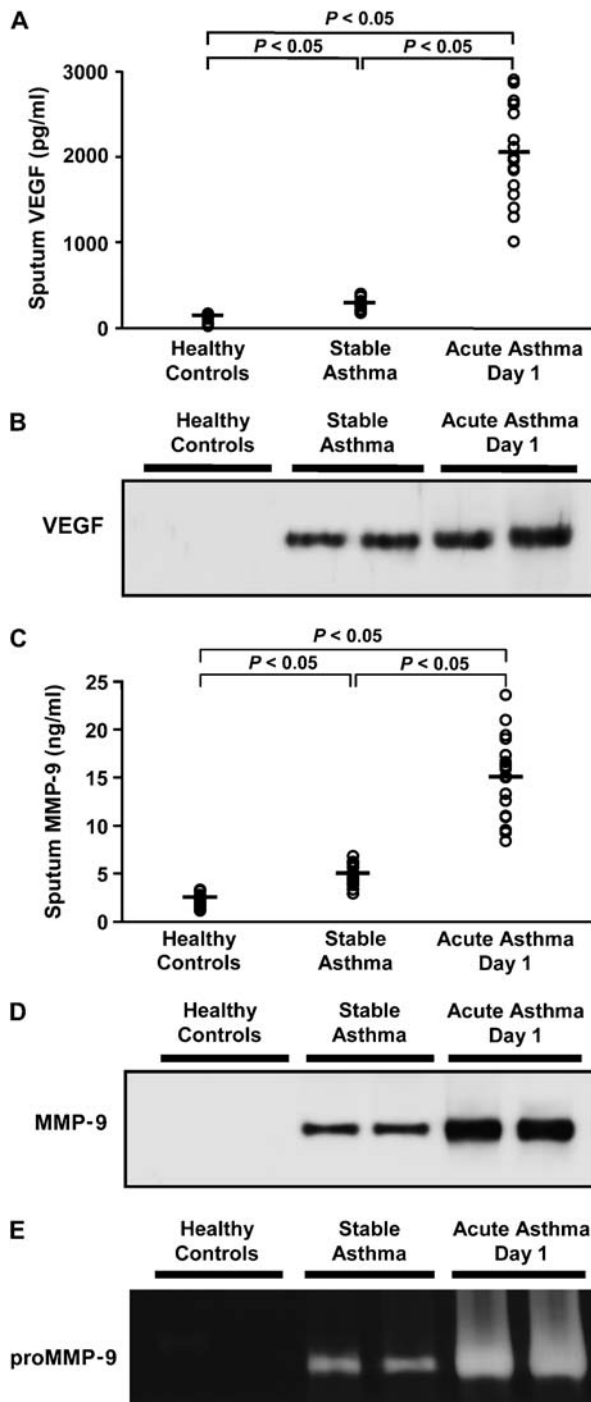


Figure 1. The levels of vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9) in sputum obtained from healthy control subjects and from subjects with stable and acute asthma. (A) Enzyme immunoassay of VEGF. (B) Western blot analysis of VEGF. (C) Enzyme immunoassay of MMP-9. (D) Western blot analysis of MMP-9. (E) Gelatin zymography of pro-MMP-9. Bars represent the mean value.

VEGF Receptor Inhibitors Decreased VEGF Protein Expression in BAL Fluids and Lung Tissues of Ovalbumin-sensitized and -challenged Mice

Levels of VEGF in BAL fluids were increased significantly at 72 h after the last challenge compared with levels after saline inhalation (Figure 4A). Administration of SU5614 or SU1498

dramatically reduced the increased levels of VEGF in BAL fluids (Figure 4A). Consistent with the results obtained from the enzyme immunoassay, Western blot analysis showed that the levels of VEGF in lung tissues were increased significantly at 72 h after the last challenge compared with levels after saline inhalation (Figures 4B and 4C). The increased levels of VEGF in lung tissues were significantly reduced by the administration of SU5614 or SU1498 (Figures 4B and 4C).

VEGF Receptor Inhibitors Reduced Plasma Extravasation in Ovalbumin-sensitized and -challenged Mice

Evans blue dye assay revealed that plasma extravasation was significantly increased at 72 h after the last challenge (Figure 5). The increase in plasma extravasation at 72 h after ovalbumin (OVA) inhalation was significantly reduced by the administration of SU5614 or SU1498.

VEGF Receptor Inhibitors Decreased MMP-9 Expression in BAL Fluids and Lung Tissues of OVA-sensitized and -challenged Mice

Levels of MMP-9 in BAL fluids were increased significantly at 72 h after the last challenge compared with levels after saline inhalation (Figure 6A). Administration of SU5614 or SU1498 dramatically reduced the increased levels of MMP-9 in BAL fluids (Figure 6A). Consistent with the results obtained from the enzyme immunoassay, Western blot analysis showed that the levels of MMP-9 in lung tissues were increased significantly at 72 h after the last challenge compared with levels after saline inhalation (Figures 6B and 6C). The increased levels of MMP-9 in lung tissues were significantly reduced by the administration of SU5614 or SU1498 (Figures 6B and 6C). In addition, zymographic analysis showed that administration of SU5614 or SU1498 decreased the increased levels of pro-MMP-9 in BAL fluids at 72 h after OVA inhalation (Figure 6D).

Immunohistochemical analysis showed localization of immunoreactive MMP-9 in inflammatory cells and epithelial layers around the bronchioles, and on inflammatory cells and debris in the airway lumen of mice with OVA-induced asthma (Figure 7B), whereas in the control mice, MMP-9 was hardly detected in inflammatory cells around the bronchioles, and on inflammatory cells and debris in the airway lumen (Figure 7A). OVA-sensitized and -challenged mice treated with SU5614 or SU1498 resulted in a decrease of immunoreactive MMP-9 localized in inflammatory cells around the bronchioles, and on inflammatory cells and debris in the airway lumen (Figures 7C and 7D).

Immunocytochemical analysis of BAL fluids showed localization of immunoreactive MMP-9 in the precipitated cells from OVA-sensitized and -challenged mice (Figure 7F). Immunoreactive MMP-9 was markedly reduced, however, in BAL cells from control mice and from OVA-sensitized and -challenged mice treated with SU5614 or SU1498 (Figures 7E, 7G, and 7H).

Effect of VEGF Receptor Inhibitors on Interleukin-4, -5, and -13 Expression

Western blot analysis revealed that interleukin (IL)-4, -5, and -13 protein levels in lung tissues were increased significantly at 72 h after OVA inhalation compared with the levels after saline inhalation (Figures 8A and 8B). The increased IL-4, IL-5, and IL-13 levels were significantly reduced by the administration of SU5614 or SU1498. Consistent with the results obtained from the Western blot analysis, enzyme immunoassays showed the significant increase in levels of IL-4, IL-5, and IL-13 in BAL fluids at 72 h after OVA inhalation as compared with the levels after saline inhalation. The increased IL-4, IL-5, and IL-13 levels

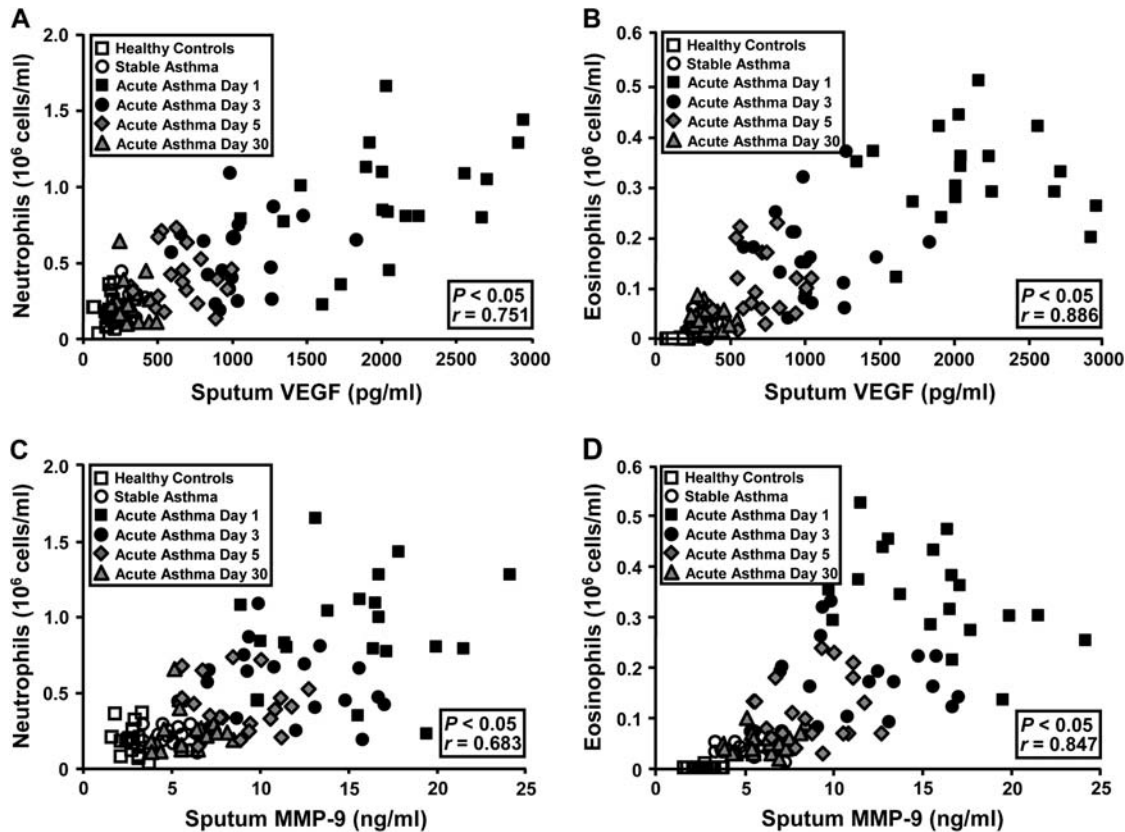


Figure 2. Correlation between the concentrations of VEGF or MMP-9 and the number of neutrophils (A and C) and eosinophils (B and D) in sputum of obtained from healthy control subjects and in subjects with stable and acute asthma.

were significantly reduced by the administration of SU5614 or SU1498 (Figure 8C).

Effect of VEGF Receptor Inhibitors on Cellular Changes in BAL Fluids

Numbers of total cells, eosinophils, lymphocytes, and neutrophils were increased significantly at 72 h after OVA inhalation compared with levels after saline inhalation (Figure 9). The increased numbers of total cells, eosinophils, lymphocytes, and neutrophils in BAL fluids were markedly reduced by the administration of the VEGF receptor inhibitors SU5614 or SU1498.

Effect of VEGF Receptor Inhibitors on Pathologic 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 (Figure 10B) as compared with the control (Figure 10A). Mice treated with SU5614 (Figure 10C) or SU1498 (Figure 10D) 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. Scores of peribronchial, perivascular, and total lung inflammation were increased significantly at 72 h after OVA inhalation compared with scores after saline inhalation (Figure 10E). The increased peribronchial, perivascular, and total lung inflammation were substantially reduced by the administration of SU5614 or SU1498. These results suggest that VEGF receptor inhibitors inhibit antigen-induced inflammation in the lungs, including the influx of eosinophils.

Effect of VEGF Receptor Inhibitors on Airway Hyperresponsiveness of OVA-induced Asthma

Airway responsiveness was assessed as a percentage increase of airway resistance (RL) in response to increasing doses of methacholine. In OVA-sensitized and -challenged mice, the dose-response curve of RL shifted to the left compared with that of control mice (Figure 11). In addition, the RL produced by methacholine administration increased significantly in the OVA-sensitized and -challenged mice compared with the control animals. OVA-sensitized and -challenged mice treated with

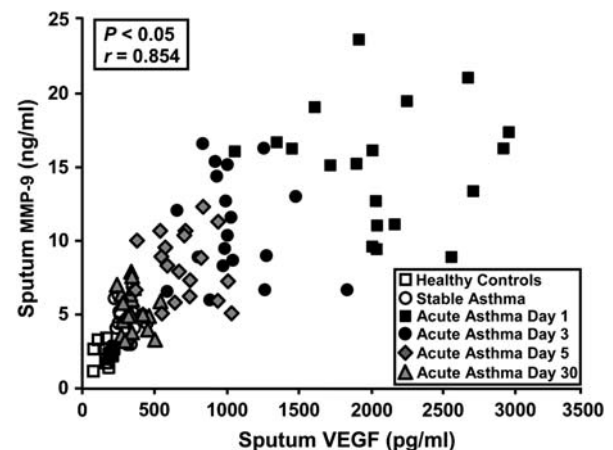


Figure 3. Correlation between the concentrations of VEGF and MMP-9 in the sputum obtained from healthy control subjects and in subjects with stable and acute asthma.

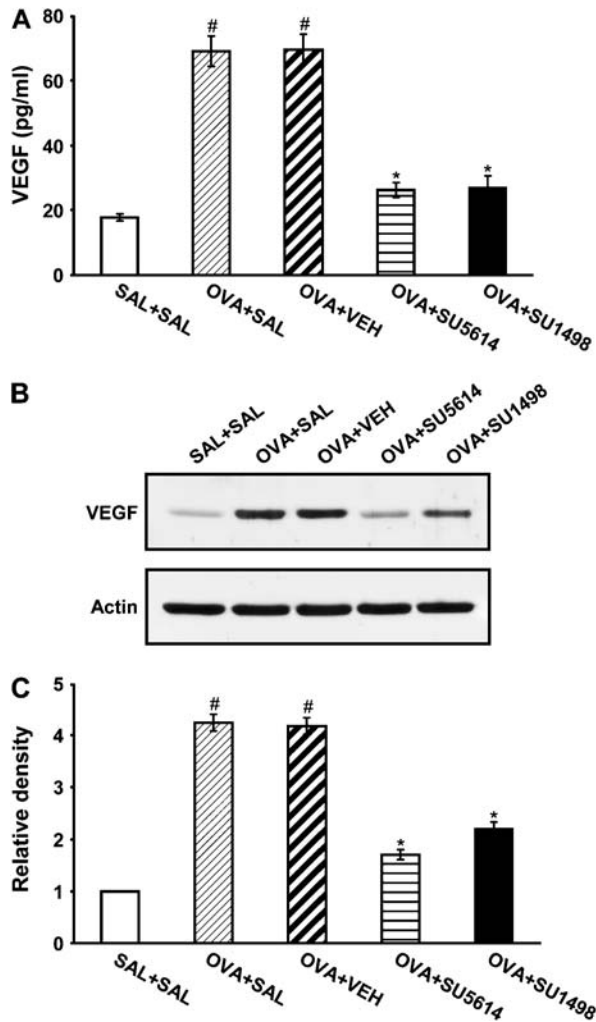


Figure 4. Effect of SU5614 or SU1498 on VEGF protein expression in bronchoalveolar lavage (BAL) fluids and lung tissues. (A) Enzyme immunoassay of VEGF in BAL fluids. Sampling was performed at 72 h after the last challenge in saline aerosol-exposed mice administered saline (SAL+Sal); ovalbumin (OVA) aerosol-exposed mice administered saline (OVA+Sal); OVA aerosol-exposed mice administered drug vehicle (0.05% dimethyl sulfoxide; OVA+VEH); OVA aerosol-exposed mice administered SU5614 (OVA+SU5614); and OVA aerosol-exposed mice administered SU1498 (OVA+SU1498). (B) Western blotting of VEGF in lung tissues. (C) Densitometric analyses are presented as the relative ratio of VEGF to actin. The relative ratio of VEGF in the lung tissues of SAL+Sal is arbitrarily presented as 1. Data represent the mean $\pm$ SEM from six mice per group. [#] $p < 0.05$ versus SAL+Sal; ^{*} $p < 0.05$ versus OVA+Sal.

SU5614 or SU1498 showed a dose-response curve of RI that shifted to the right compared with that of untreated mice. These results indicate that VEGF receptor inhibitor treatment reduces OVA-induced airway hyperresponsiveness.

DISCUSSION

VEGF is a potent stimulator of inflammation, airway remodeling, and physiologic dysregulation that augments antigen sensitization and Th2-cell inflammation (19). MMP-9 plays a role in chronic airway inflammation and remodeling in asthma (20–25). Few data are available, however, on a potential relationship between VEGF and MMP-9 in the pathogenesis of asthma. This study demonstrates that levels of VEGF and MMP-9 are signifi-

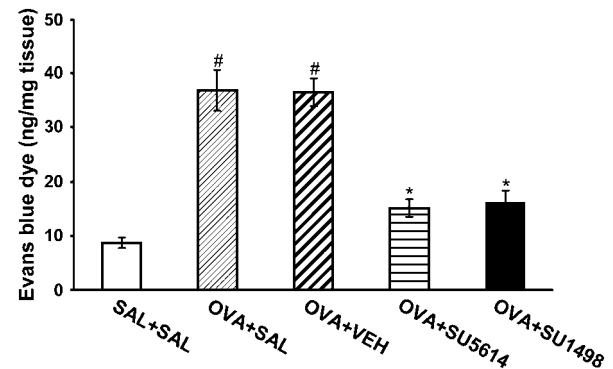


Figure 5. Effect of SU5614 or SU1498 on plasma exudation in OVA-sensitized and -challenged mice. Mice were treated as described in Figure E1. Sampling was performed at 72 h after the last challenge in saline aerosol-exposed mice administered saline (SAL+Sal); OVA aerosol-exposed mice administered saline (OVA+Sal); OVA aerosol-exposed mice administered drug vehicle (OVA+VEH); OVA aerosol-exposed mice administered SU5614 (OVA+SU5614); and OVA aerosol-exposed mice administered SU1498 (OVA+SU1498). Data represent the mean $\pm$ SEM from six mice per group. [#] $p < 0.05$ versus SAL+Sal; ^{*} $p < 0.05$ versus OVA+Sal.

cantly higher in the sputum of patients with asthma than in healthy control subjects, and a significant correlation is found between VEGF and MMP-9 levels. These findings with human subjects have further evaluated using a murine model of asthma induced by OVA. The results have revealed that the asthma model exhibits typical pathophysiologic features of asthma in the lungs but also the increase in the levels of VEGF and MMP-9. Moreover, administration of VEGF receptor inhibitors reduces the pathophysiologic signs of asthma and the increased expression of MMP-9. Taken together, these observations have suggested that VEGF signaling regulates MMP-9 expression and plays an important role in initiation and upholding of asthmatic signs.

Bronchial asthma is characterized by inflammation of the airways, which is usually accompanied by increased vascular permeability, resulting in plasma exudation (32). Exudation of plasma proteins into the airways plays a pivotal role in airway inflammation, hyperresponsiveness, and asthmatic tissue edema (13, 14, 33). A number of studies have indicated that expression of VEGF is increased in asthma and overproduction of VEGF can increase vascular permeability so that plasma proteins, including inflammatory mediators, and inflammatory cells can leak into the extravascular space to allow the migration of inflammatory cells into the airways (7, 9, 14, 17–19, 34, 35). The present study with human subjects has revealed that the levels of VEGF are significantly increased in patients with stable asthma and even higher in patients with acute asthma compared with healthy control subjects. The levels of VEGF in the sputum of patients with asthma during exacerbation were significantly higher compared with remission days, and those levels decreased after asthma therapy. In addition, the levels of VEGF in sputum were closely correlated with the number of neutrophils and eosinophils. Consistent with these observations, we have found that VEGF expression is up-regulated in OVA-induced asthma. Moreover, administration of VEGF receptor inhibitors SU5614 or SU1498 decreases bronchial hyperresponsiveness, airway inflammation, the increased plasma exudation, and the overexpression of VEGF. The chemical inhibitors may inhibit other pathways nonspecifically. SU5614 is a potent inhibitor of VEGF and

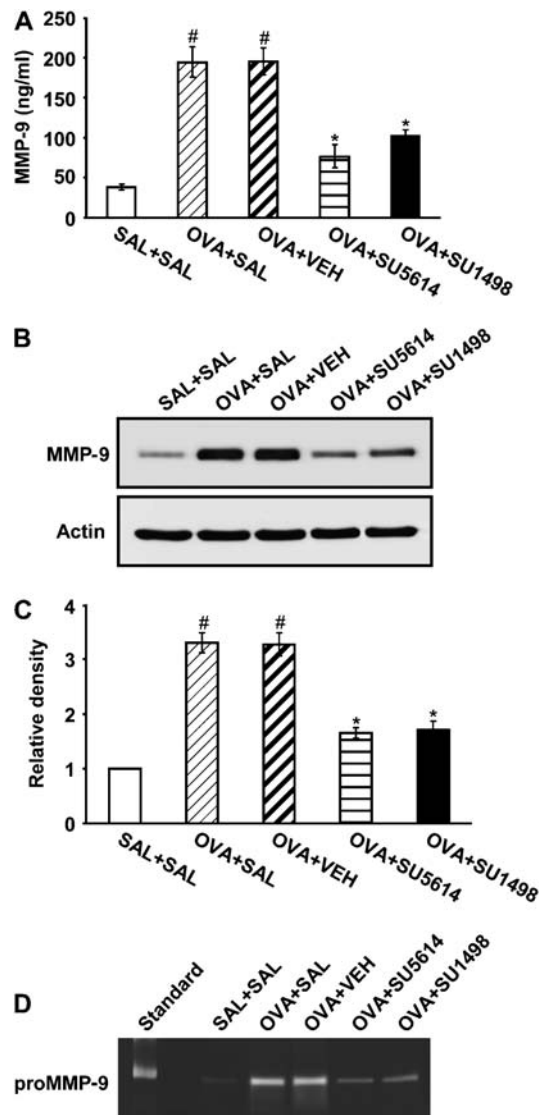


Figure 6. Effect of SU5614 or SU1498 on MMP-9 protein expression in BAL fluids and lung tissues. (A) Enzyme immunoassay of MMP-9 in BAL fluids. Sampling was performed at 72 h after the last challenge in saline aerosol-exposed mice administered saline (SAL+Sal); OVA aerosol-exposed mice administered saline (OVA+Sal); OVA aerosol-exposed mice administered drug vehicle (OVA+VEH); OVA aerosol-exposed mice administered SU5614 (OVA+SU5614); and OVA aerosol-exposed mice administered SU1498 (OVA+SU1498). (B) Western blotting of MMP-9 in lung tissues. (C) Densitometric analyses are presented as the relative ratio of MMP-9 to actin. The relative ratio of MMP-9 in the lung tissues of SAL+Sal is arbitrarily presented as 1. (D) Gelatin zymography of pro-MMP-9. Data represent the mean $\pm$ SEM from six mice per group. # $p < 0.05$ versus SAL+Sal; * $p < 0.05$ versus OVA+Sal.

platelet-derived growth factor receptor tyrosine kinases. It does not have any effect on the epidermal growth factor and insulin-like growth factor receptor tyrosine kinases. Previous studies have demonstrated that SU5614 also has inhibitory activity on other receptors, such as c-kit and Flt-3 (36–38). In addition, SU1498 is a potent and selective inhibitor of Flk-1 kinase, a VEGF receptor kinase. It has only a weak inhibitory effect on platelet-derived growth factor receptor, epidermal growth factor receptor, and HER-2 kinase. The use of these two VEGF receptor inhibitors may rule out the inhibitory effects of these chemicals on other receptors except for VEGF receptor tyrosine ki-

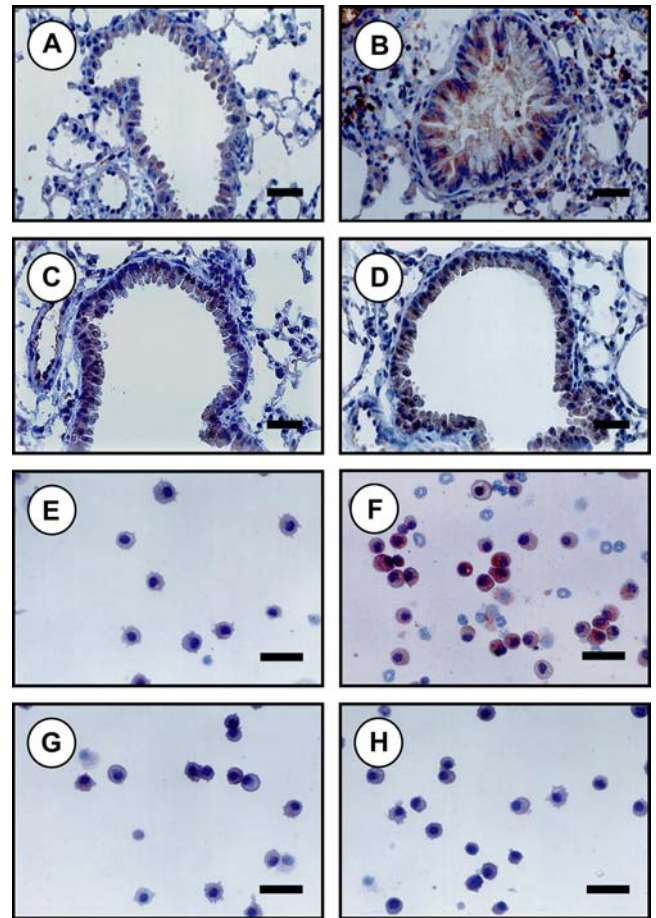


Figure 7. Localization of immunoreactive MMP-9 in lung tissues and BAL fluids of OVA-sensitized and -challenged mice. Sampling was performed at 72 h after the last challenge in saline aerosol-exposed mice administered saline (A and E); OVA aerosol-exposed mice administered saline (B and F); OVA aerosol-exposed mice administered SU5614 (C and G); and OVA aerosol-exposed mice administered SU1498 (D and H). (A–D) Representative light microscopy of MMP-9-positive cells in the bronchioles; the brown color indicates MMP-9-positive cells. (E–H) Representative light microscopy of MMP-9-positive cells in the BAL fluids; the dark brown color indicates MMP-9-positive cells. Bars indicate scale of 50 μ m.

nase. In the present study, the plasma leakage and the migration of inflammatory cells, which are the sources of VEGF production, were blocked by administration of SU5614 or SU1498, thereby inhibiting VEGF production. In addition, recent studies have demonstrated that there is a positive feedback loop, with VEGF enhancing Th2 sensitization, inflammation, and cytokine elaboration, and IL-13 subsequently enhancing VEGF production (19, 39). The data have also shown that inhibition of VEGF activity by administration of VEGF receptor inhibitors decreases Th2 inflammation and cytokine elaboration in OVA-sensitized and -challenged mice, leading to inhibition of VEGF production. Vega-Diaz and coworkers (40) have reported that the autocrine action of VEGF seems to be mediated by the KDR receptor and anti-KDR antibody blocks the VEGF message. Taken together, these findings suggest that VEGF receptor inhibitors decrease VEGF production through inhibition of migration and inflammatory responses of VEGF-producing cells or blocking of auto-inductive VEGF pathway, and VEGF may play a crucial role in the pathogenesis of patients with asthma.

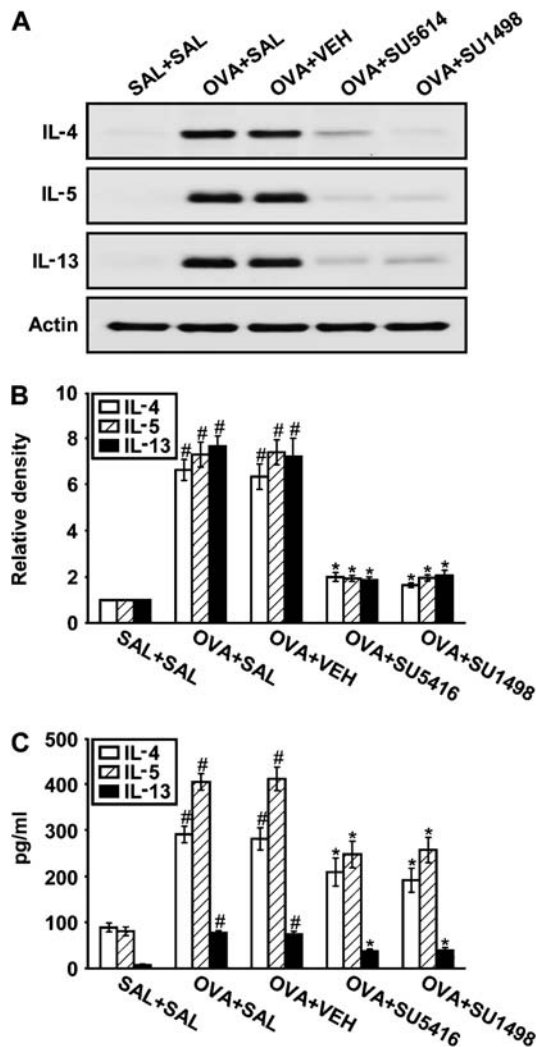


Figure 8. Effect of SU5614 or SU1498 on interleukin (IL)-4, IL-5, and IL-13 in lung tissues and in BAL fluids of OVA-sensitized and -challenged mice. Sampling was performed at 72 h after the last challenge in saline aerosol-exposed mice administered saline (SAL+SAL); OVA aerosol-exposed mice administered saline (OVA+SAL); OVA aerosol-exposed mice administered drug vehicle (OVA+VEH); OVA aerosol-exposed mice administered SU5614 (OVA+SU5614); and OVA aerosol-exposed mice administered SU1498 (OVA+SU1498). (A) Western blotting of IL-4, IL-5, and IL-13 in lung tissues. (B) Densitometric analyses are presented as the relative ratio of each molecule to actin. The relative ratio of each molecule in the lung tissues of SAL+SAL is arbitrarily presented as 1. (C) Enzyme immunoassay of IL-4, IL-5, and IL-13 in BAL fluids. Bars represent the mean $\pm$ SEM from six mice per group. [#] $p < 0.05$ versus SAL+SAL; ^{*} $p < 0.05$ versus OVA+SAL.

A balance between expression of proteases and antiproteases, particularly MMPs and their inhibitors, maintains homeostasis between synthesis and degradation of extracellular matrix components under normal conditions. The expression of these proteases and antiproteases is critical in tissue repair and remodeling in some pulmonary inflammatory diseases (41–44). Previous studies have demonstrated that MMP-9 may play a role in chronic airway inflammation, including induction of migration of eosinophils, lymphocytes, and neutrophils across basement membranes, and remodeling in asthma (20–25). Recently, we have demonstrated that MMP-9 plays a crucial role in the infiltration of airway inflammatory cells and the induction of airway hyperresponsiveness in toluene diisocyanate-induced asthma.

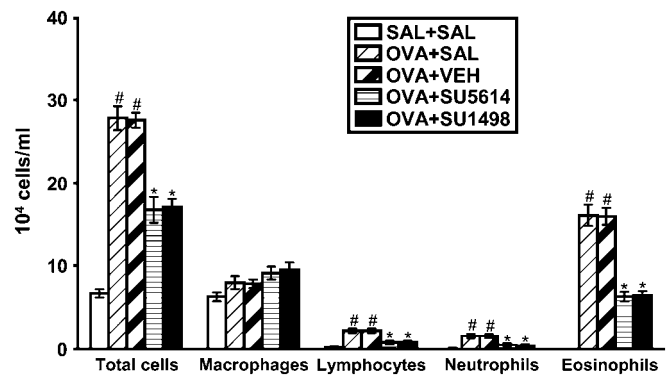


Figure 9. Effect of SU5614 or SU1498 on total cells and differential cellular components in BAL fluids of OVA-sensitized and -challenged mice. The numbers of total cells and differential cellular components of BAL from saline aerosol-exposed mice administered saline (SAL+SAL), OVA aerosol-exposed mice administered saline (OVA+SAL), OVA aerosol-exposed mice administered drug vehicle (OVA+VEH), OVA aerosol-exposed mice administered SU5614 (OVA+SU5614), and OVA aerosol-exposed mice administered SU1498 (OVA+SU1498) were counted at 72 h after the last challenge. Bars represent the mean $\pm$ SEM from six mice per group. [#] $p < 0.05$ versus SAL+SAL; ^{*} $p < 0.05$ versus OVA+SAL.

The inhibition of MMP-9 may be a good therapeutic strategy (45–47). The role of MMP-9 in asthmatic airways, however, is still controversial. A recent study using MMP-9-deficient mice in an allergen-challenged model has demonstrated heightened inflammation and airway responsiveness and suggested the role of MMP-9 in the resolution of lung injury after antigen challenge (48). In this study, the authors found that the levels of MMP-9 are significantly increased in patients with stable asthma and even higher in patients with acute asthmatic patients compared with healthy control subjects. The levels of MMP-9 in the sputum of patients with asthma during exacerbation were significantly higher compared with remission days, and those levels decreased after asthma therapy. Moreover, the levels of MMP-9 in sputum were significantly correlated with the number of neutrophils and eosinophils. These findings indicate that MMP-9 may have an important role in inducing and maintaining asthma.

In our study, a significant correlation has been found between VEGF and MMP-9 levels in the sputum of healthy control subjects, patients with stable asthma, and patients with acute asthma. Reports showing a potential role of MMP-9 in pathogenesis of the diseases have accumulated over the years, and it is now well established that VEGF plays a critical role in asthma (7, 19). Recently, Valable and coworkers (49) have shown that VEGF-induced permeability is associated with the increase of an MMP-9 activity in *in vivo* and *in vitro* models. Based on these observations, we administered the VEGF receptor inhibitors SU5614 or SU1498 to examine their potential therapeutic effects and to evaluate the effect of VEGF receptor inhibitors on MMP-9 expression. The VEGF receptor inhibitors were effective at reversing all pathophysiologic signs examined and reduced the increased expression of MMP-9. One likely mechanism for the effectiveness of SU5614 or SU1498 is that VEGF can increase vascular permeability so that plasma proteins and inflammatory cells, which are the sources of MMP-9, can leak into the extravascular space to allow the migration of inflammatory cells, including eosinophils, into the airways. The inhibition of the plasma leakage and the migration of inflammatory cells by administration of SU5614 or SU1498 resulted in reduction of MMP-9 production. In addition, the MMP family has several

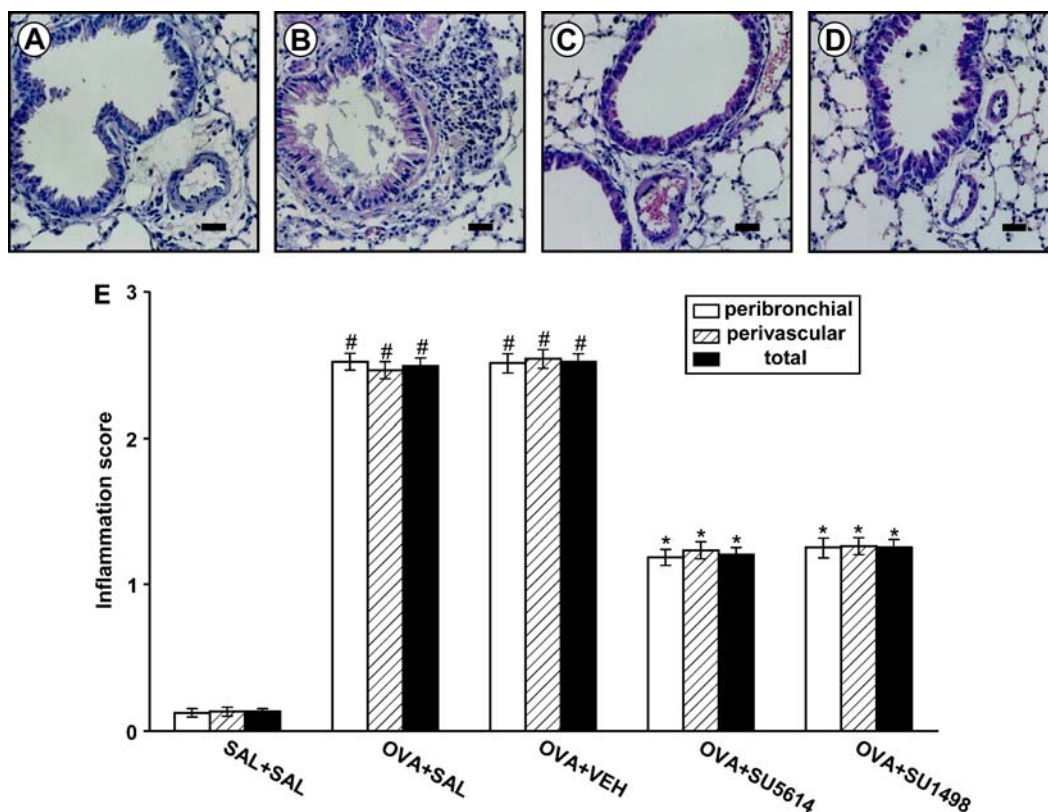


Figure 10. Effect of SU5614 or SU1498 on pathologic changes in lung tissues of OVA-sensitized and -challenged mice. (A–D) Representative hematoxylin-and-eosin-stained sections of the lungs. Sampling was performed at 72 h after the last challenge in saline aerosol-exposed mice administered saline (A); OVA aerosol-exposed mice administered saline (B); OVA aerosol-exposed mice administered SU5614 (C); and OVA aerosol-exposed mice administered SU1498 (D). Bars indicate scale of 50 μ m. (E) Peribronchial and perivascular lung inflammation were measured at 72 h after the last challenge in saline aerosol-exposed mice administered saline (SAL+Sal); OVA aerosol-exposed mice administered saline (OVA+Sal); OVA aerosol-exposed mice administered drug vehicle (OVA+VEH); OVA aerosol-exposed mice administered SU5614 (OVA+SU5614); and OVA aerosol-exposed mice administered SU1498 (OVA+SU1498). Total lung inflammation was defined as the average of the peribronchial and perivascular inflammation scores. Bars represent the mean $\pm$ SEM from six mice per group. [#]p < 0.05 versus SAL+Sal; ^{*}p < 0.05 versus OVA+Sal.

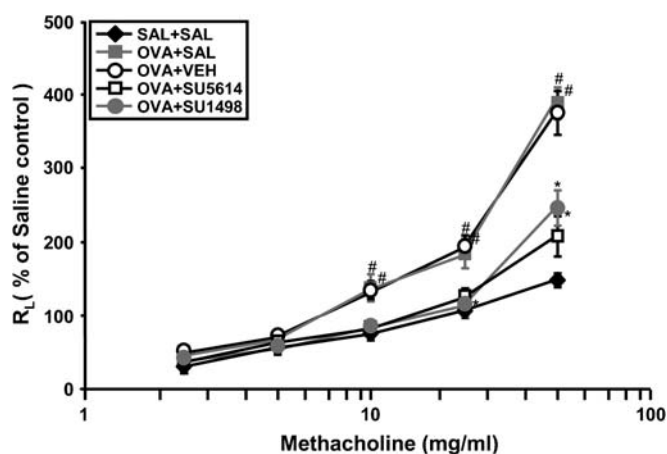


Figure 11. Effect of SU5614 or SU1498 on airway responsiveness of OVA-sensitized and -challenged mice. Airway responsiveness was measured at 72 h after the last challenge in saline aerosol-exposed mice administered saline (SAL+Sal); OVA aerosol-exposed mice administered saline (OVA+Sal); OVA aerosol-exposed mice administered drug vehicle (OVA+VEH); OVA aerosol-exposed mice administered SU5614 (OVA+SU5614); and OVA aerosol-exposed mice administered SU1498 (OVA+SU1498). RL values were obtained in response to increasing doses (2.5–50 mg/ml) of methacholine as described in METHODS. Bars represent the mean $\pm$ SEM from six mice per group. [#]p < 0.05 versus SAL+Sal; ^{*}p < 0.05 versus OVA+Sal.

points of interaction with the cytokine network. Inflammatory cytokines and growth factors, such as IL-13, tumor necrosis factor α , IL-1 β , and IL-8, can regulate MMP-9 expression (50–53). We have found that the increased IL-13 levels after OVA challenge were significantly reduced by the administration of SU5614 or SU1498. These results suggest that VEGF receptor inhibition reduces the specific cellular expression of MMP-9 by leukocytes through reducing inflammatory cytokines. These observations strongly indicate that the inhibition of VEGF signaling is a potentially powerful therapeutic strategy for asthma, partly mediated through regulation of MMP-9. These findings provide an important mechanism for the use of VEGF receptor inhibitors to prevent or treat asthma and other airway inflammatory disorders.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: The authors thank Professor Mie-Jae Im for careful reading of the manuscript.

References

1. Roche WR, Beasley R, Williams JH, Holgate ST. Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* 1989;1:520–524.
2. Wiggs BR, Bosken C, Pare PD, James A, Hogg JC. A model of airway narrowing in asthma and in chronic obstructive pulmonary disease. *Am Rev Respir Dis* 1992;145:1251–1258.
3. Cotran RS, Kumar V, Collins T, Robbins SL. Inflammation and repair. In: Cotran RS, Kumar V, Collins T, Robbins SL, editors. Robbins pathologic basis of disease, 6th ed. Philadelphia: W.B. Saunders; 1989. pp. 39–87.

4. Vrugt B, Wilson S, Bron A, Holgate ST, Djukanovic R, Aalbers R. Bronchial angiogenesis in severe glucocorticoid-dependent asthma. *Eur Respir J* 2000;15:1014–1021.
5. Salvato G. Quantitative and morphological analysis of the vascular bed in bronchial biopsy specimens from asthmatic and non-asthmatic subjects. *Thorax* 2001;56:902–906.
6. Li X, Wilson JW. Increased vascularity of the bronchial mucosa in mild asthma. *Am J Respir Crit Care Med* 1997;156:229–233.
7. Lee YC, Lee HK. Vascular endothelial growth factor in patients with acute asthma. *J Allergy Clin Immunol* 2001;107:1106.
8. Orsida BE, Ward C, Li X, Bish R, Wilson JW, Thien F, Walters EH. Effect of a long-acting β_2 -agonist over three months on airway wall vascular remodeling in asthma. *Am J Respir Crit Care Med* 2001;164:117–121.
9. Hoshino M, Nakamura Y, Hamid QA. Gene expression of vascular endothelial growth factor and its receptors and angiogenesis in bronchial asthma. *J Allergy Clin Immunol* 2001;107:1034–1038.
10. Hoshino M, Takahashi M, Aoike N. Expression of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin immunoreactivity in asthmatic airways and its relationship to angiogenesis. *J Allergy Clin Immunol* 2001;107:295–301.
11. Kanazawa H, Hirata K, Yoshikawa J. Involvement of vascular endothelial growth factor in exercise induced bronchoconstriction in asthmatic patients. *Thorax* 2002;57:885–888.
12. Asai K, Kanazawa H, Otani K, Shiraiishi S, Hirata K, Yoshikawa J. Imbalance between vascular endothelial growth factor and endostatin levels in induced sputum from asthmatic subjects. *J Allergy Clin Immunol* 2002;110:571–575.
13. Charan NB, Baile EM, Pare PD. Bronchial vascular congestion and angiogenesis. *Eur Respir J* 1997;10:1173–1180.
14. Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, Glazer N, Holash J, McDonald DM, Yancopoulos GD. Angiopoietin-1 protects the adult vasculature against plasma leakage. *Nat Med* 2000;6:460–463.
15. Kanazawa H, Nomura S, Yoshikawa J. Role of microvascular permeability on physiologic differences in asthma and eosinophilic bronchitis. *Am J Respir Crit Care Med* 2004;169:1125–1130.
16. Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 1995;146:1029–1039.
17. Lee YC, Kwak YG, Song CH. Contribution of vascular endothelial growth factor to airway hyperresponsiveness and inflammation in a murine model of toluene diisocyanate-induced asthma. *J Immunol* 2002;168:3595–3600.
18. Lee KS, Kim SR, Park HS, Jin GY, Lee YC. Cysteinyl leukotriene receptor antagonist regulates vascular permeability by reducing vascular endothelial growth factor expression. *J Allergy Clin Immunol* 2004;114:1093–1099.
19. Lee CG, Link H, Baluk P, Homer RJ, Chapoval S, Bhandari V, Kang MJ, Cohn L, Kim YK, McDonald DM, et al. Vascular endothelial growth factor (VEGF) induces remodeling and enhances T_H2 -mediated sensitization and inflammation in the lung. *Nat Med* 2004;10:1095–1103.
20. Vignola AM, Riccobono L, Mirabella A, Profita M, Chanez P, Bellia V, Mautino G, D'acardi P, Bousquet J, Bonsignore G. Sputum metalloproteinase-9/tissue inhibitor of metalloproteinase-1 ratio correlates with airflow obstruction in asthma and chronic bronchitis. *Am J Respir Crit Care Med* 1998;158:1945–1950.
21. Hoshino M, Takahashi M, Takai Y, Sim J. Inhaled corticosteroids decrease subepithelial collagen deposition by modulation of the balance between matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 expression in asthma. *J Allergy Clin Immunol* 1999;104:356–363.
22. Simpson JL, Scott RJ, Boyle MJ, Gibson PG. Differential proteolytic enzyme activity in eosinophilic and neutrophilic asthma. *Am J Respir Crit Care Med* 2005;172:559–565.
23. Okada S, Kita H, George TJ, Gleich GJ, Leiferman KM. Migration of eosinophils through basement membrane components *in vitro*: role of matrix metalloproteinase-9. *Am J Respir Cell Mol Biol* 1997;17:519–528.
24. Leppert D, Waubant E, Galardy R, Bunnett NW, Hauser SL. T cell gelatinases mediate basement membrane transmigration *in vitro*. *J Immunol* 1995;154:4379–4389.
25. Delclaux C, Delacourt C, D'Ortho MP, Boyer V, Lafuma C, Harf A. Role of gelatinase B and elastase in human polymorphonuclear neutrophil migration across basement membrane. *Am J Respir Cell Mol Biol* 1996;14:288–295.
26. Lee KS, Min KH, Kim SR, Park SJ, Park HS, Jin GY, Lee YC. Vascular endothelial growth factor modulates matrix metalloproteinase-9 expression in asthma [abstract; in Korean]. *Tuberc Respir Dis* 2005;59:199.
27. Fahy JV, Steiger DJ, Liu J, Basbaum CB, Finkbeiner WE, Boushey HA. Markers of mucus secretion and DNA levels in induced sputum from asthmatic and from healthy subjects. *Am Rev Respir Dis* 1993;147:1132–1137.
28. Lee YC, Lee HB, Rhee YK, Song CH. The involvement of matrix metalloproteinase-9 in airway inflammation of patients with acute asthma. *Clin Exp Allergy* 2001;31:1623–1630.
29. Kwak YG, Song CH, Yi HK, Hwang PH, Kim JS, Lee KS, Lee YC. Involvement of PTEN in airway hyperresponsiveness and inflammation in bronchial asthma. *J Clin Invest* 2003;111:1083–1092.
30. Tournay KG, Kips JC, Schou C, Pauwels RA. Airway eosinophilia is not a requirement for allergen-induced airway hyperresponsiveness. *Clin Exp Allergy* 2000;30:79–85.
31. Takeda K, Hamelmann E, Joetham A, Shultz LD, Larsen GL, Irvin CG, Gelfand EW. Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice. *J Exp Med* 1997;186:449–454.
32. Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma: from bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* 2000;161:1720–1745.
33. Antony AB, Tepper RS, Mohammed KA. Cockroach extract antigen increases bronchial airway epithelial permeability. *J Allergy Clin Immunol* 2002;110:589–595.
34. Horiuchi H, Weller PF. Expression of vascular endothelial growth factor by human eosinophils: upregulation by granulocyte macrophage colony stimulating factor and interleukin-5. *Am J Respir Cell Mol Biol* 1997;17:70–77.
35. Taichman NS, Young S, Cruchley AT, Taylor P, Paleolog E. Human neutrophils secrete vascular endothelial growth factor. *J Leukoc Biol* 1997;62:397–400.
36. Spiekermann K, Dirschinger RJ, Schwab R, Bagrintseva K, Faber F, Buske C, Schnittger S, Kelly LM, Gilliland G, Hiddemann W. The protein tyrosine kinase inhibitor SU5614 inhibits FLT3 and induces growth arrest and apoptosis in AML-derived cell lines expressing a constitutively activated FLT3. *Blood* 2003;101:1494–1504.
37. Yee KW, O'Farrell AM, Smolich BD, Cherrington JM, McMahon G, Wait CL, McGreevey LS, Griffith DJ, Heinrich MC. SU5614 and SU5614 inhibit kinase activity of wild-type and mutant FLT3 receptor tyrosine kinase. *Blood* 2002;100:2941–2949.
38. Spiekermann K, Faber F, Voswinckel R, Hiddemann W. The protein tyrosine kinase inhibitor SU5614 inhibits VEGF-induced endothelial cell sprouting and induces growth arrest and apoptosis by inhibition of c-kit in AML cells. *Exp Hematol* 2002;30:767–773.
39. Corne J, Chupp G, Lee CG, Homer RJ, Zhu Z, Chen Q, Ma B, Du Y, Roux F, McArdle J, et al. IL-13 stimulates vascular endothelial cell growth factor and protects against hyperoxic acute lung injury. *J Clin Invest* 2000;106:783–791.
40. Vega-Diaz B, Herron GS, Michel S. An autocrine loop mediates expression of vascular endothelial growth factor in human dermal microvascular endothelial cells. *J Invest Dermatol* 2001;116:525–530.
41. Delacourt C, Le Bourgeois M, D'Ortho MP, Doit C, Scheinmann P, Navarro J, Harf A, Hartmann DJ, Lafuma C. Imbalance between 95 kDa type IV collagenase and tissue inhibitor of metalloproteinases in sputum of patients with cystic fibrosis. *Am J Respir Crit Care Med* 1995;152:765–774.
42. Hayashi T, Stetler-Stevenson WG, Fleming MV, Fishback N, Koss MN, Liotta LA, Ferrans VJ, Travis WD. Immunohistochemical study of metalloproteinases and their tissue inhibitors in the lungs of patients with diffuse alveolar damage and idiopathic pulmonary fibrosis. *Am J Pathol* 1996;149:1241–1256.
43. Ricou B, Nicod L, Lacraz S, Welgus HG, Suter PM, Dayer JM. Matrix metalloproteinases and TIMP in acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1996;154:346–352.
44. Sepper R, Kontinen YT, Ding Y, Takagi M, Sorsa T. Human neutrophil collagenase (MMP-8), identified in bronchiectasis BAL fluid, correlates with severity of disease. *Chest* 1995;107:1641–1647.
45. Lee YC, Song CH, Lee HB, Oh JL, Rhee YK, Park HS, Koh GY. A murine model of toluene diisocyanate-induced asthma can be treated with matrix metalloproteinase inhibitor. *J Allergy Clin Immunol* 2001;108:1021–1026.
46. Lee KS, Jin SM, Kim HJ, Lee YC. Matrix metalloproteinase inhibitor regulates inflammatory cell migration by reducing ICAM-1 and

- VCAM-1 expression in a murine model of toluene diisocyanate-induced asthma. *J Allergy Clin Immunol* 2003;111:1278–1284.
47. Lee KS, Jin SM, Kim SS, Lee YC. Doxycycline reduces airway inflammation and hyperresponsiveness in a murine model of toluene diisocyanate-induced asthma. *J Allergy Clin Immunol* 2004;113:902–909.
48. McMillan SJ, Kearley J, Campbell JD, Zhu XW, Larbi KY, Shipley JM, Senior RM, Nourshargh S, Lloyd CM. Matrix metalloproteinase-9 deficiency results in enhanced allergen-induced airway inflammation. *J Immunol* 2004;172:2586–2594.
49. Valable S, Montaner J, Bellail A, Berezowski V, Brillault J, Cecchelli R, Divoux D, Mackenzie ET, Bernaudin M, Roussel S, *et al.* VEGF-induced BBB permeability is associated with an MMP-9 activity increase in cerebral ischemia: both effects decreased by Ang-1. *J Cereb Blood Flow Metab* 2005;25:1491–1504.
50. Schwingshackl A, Duszyk M, Brown N, Moqbel R. Human eosinophils release matrix metalloproteinase-9 on stimulation with TNF- α . *J Allergy Clin Immunol* 1999;104:983–989.
51. Yao PM, Maitre B, Delacourt C, Buhler JM, Harf A, Lafuma C. Divergent regulation of 92-kDa gelatinase and TIMP-1 by HBECs in response to IL-1 β and TNF- α . *Am J Physiol* 1997;273:L866–L874.
52. Van den Steen PE, Proost P, Wuyts A, Van Damme J, Opdenakker G. Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO- α and leaves RANTES and MCP-2 intact. *Blood* 2000;96:2673–2681.
53. Lanone S, Zheng T, Zhu Z, Liu W, Lee CG, Ma B, Chen Q, Homer RJ, Wang J, Rabach LA, *et al.* Overlapping and enzyme-specific contributions of matrix metalloproteinases-9 and -12 in IL-13-induced inflammation and remodeling. *J Clin Invest* 2002;110:463–474.