

# A potent antiangiogenic factor, endostatin prevents the development of asthma in a murine model

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**Background:** Clinical studies suggest a role for angiogenesis in the development and persistence of chronic asthma, but whether angiogenic mediators contribute to acute asthma has not been fully studied.

**Objective:** The aim of this study was to investigate a role of vascular endothelial growth factor (VEGF), a major angiogenic and proinflammatory mediator, in allergen-induced acute asthma and to determine whether endostatin/Fc, a potent antiangiogenic factor can attenuate allergic airway responses. **Methods:** We sensitized BALB/c mice with ovalbumin. We measured serum VEGF and examined immunoreactive VEGF around the airways 48 hours after the last challenge with either aerosolized PBS or ovalbumin once per day for 3 days. We also treated ovalbumin-sensitized mice with either endostatin/Fc or control fusion protein at the time of challenge with ovalbumin. We analyzed allergic airway responses 48 hours after the last ovalbumin challenge.

**Results:** Ovalbumin challenge induced immunolocalization of numerous VEGF-positive cells around airways and increased serum VEGF levels. Treatment with endostatin/Fc inhibited the airway hyperresponsiveness, pulmonary allergic inflammation, production of ovalbumin-specific IgE, and lung inflammatory mediators. Both VEGF-dependent and independent mechanisms are indicated by results using antibody blockade of VEGF receptors, which caused decreased allergic pulmonary inflammation but did not alter airway hyperresponsiveness or serum IgE levels.

**Conclusion:** These data demonstrate for the first time that recombinant endostatin can prevent the development of asthma features in a mouse model and suggest that this class of agents

merits further study as novel therapeutics for asthma. (*J Allergy Clin Immunol* 2005;116:1220-7.)

**Key words:** Endostatin, vascular endothelial growth factor, ovalbumin, asthma

The incidence and prevalence of asthma have markedly risen in industrialized countries.<sup>1</sup> Despite advances in understanding of the inflammatory and immunologic components of asthma, more progress is needed in development of novel therapies. Current treatments have been designed to suppress the inflammatory response for therapeutic benefit.<sup>2</sup> Although some success has been achieved with the use of anti-inflammatory drugs such as corticosteroids and leukotriene receptor antagonists, the structural changes in established asthma are poorly responsive to steroid-based therapy.<sup>3,4</sup> In addition, remodeling changes may begin early in the development of asthma and might occur in parallel with inflammation or even be required for the establishment of persistent inflammation.<sup>5</sup> For example, airway biopsy studies reveal that tissue restructuring is already present in childhood asthma well before the ravages of chronic inflammation would be expected.<sup>6</sup> Hence, strategies to suppress both inflammatory responses and airway remodeling would be especially promising.

Angiogenesis, the growth and proliferation of new blood vessels, is a critical process in both physiologic and pathologic responses in a variety of disease conditions including wound repair, tumor growth, and rheumatoid arthritis.<sup>7-10</sup> Although little is known about the role of angiogenesis in asthma, several studies have shown an increase of vessels and vascularity (the percentage of lamina propria occupied by vessels) in bronchial mucosa taken from patients with asthma in comparison with normal mucosa.<sup>11-13</sup> These morphometric observations indicate that angiogenesis contributes to the process of airway remodeling in patients with asthma. Vascular endothelial growth factor (VEGF) is a potent angiogenic factor and also acts as a proinflammatory cytokine by increasing endothelial permeability and inducing endothelial adhesion molecules.<sup>14-16</sup> VEGF-positive cells are significantly increased in number in the airway mucosa of subjects with asthma compared with numbers seen in healthy control subjects.<sup>17</sup>

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#### Abbreviations used

|                  |                                             |
|------------------|---------------------------------------------|
| AHR:             | Airway hyperresponsiveness                  |
| AI:              | Allergic pulmonary inflammation             |
| BAL:             | Bronchoalveolar lavage                      |
| BALF:            | Bronchoalveolar lavage fluid                |
| MCP-1:           | Monocyte chemoattractant protein 1          |
| MIP-1 $\alpha$ : | Macrophage inflammatory protein 1 $\alpha$  |
| Penh:            | Enhanced pause                              |
| VEGF:            | Vascular endothelial growth factor          |
| VEGFR:           | Vascular endothelial growth factor receptor |

The data suggest that VEGF might play an important role in the development and persistence of asthma as both a proinflammatory cytokine and as an angiogenic factor.

We hypothesized that antiangiogenic therapy could block the development of asthma by inhibiting both inflammation and airway remodeling. We began our investigation by testing whether VEGF contributed to the development of allergen-induced acute asthma. Next we tested whether antiangiogenic therapy could attenuate acute allergic airway responses. For this purpose, we used murine endostatin/Fc, a potent antiangiogenic factor that inhibits endothelial cell proliferation,<sup>18</sup> migration,<sup>19</sup> and angiogenesis in response to VEGF.<sup>20-23</sup> Because endostatin dramatically prevented physiologic and pathologic features of asthma in our model, we further investigated the role of VEGF by using receptor blockade interventions. The data support both VEGF-dependent and independent mechanisms for a remarkably potent antiasthmatic effect of endostatin.

## METHODS

### Animals

Female BALB/c mice 6 to 8 weeks old were purchased from Japan SLC (Shizuoka, Japan). The mice were housed in the animal facility of Nara Medical University that was maintained at 22–24°C with a 12-hour dark/light cycle. Mice were fed a commercial pelleted mouse food and given water ad libitum under specific pathogen-free conditions according to standard guidelines for the care and use of animals.<sup>24</sup> All experiments were conducted under protocols approved by our institutional review board.

### Allergen sensitization and exposure

The mice were sensitized by initial i.p. injection of 0.4 mL phosphate buffered saline (PBS) containing ovalbumin (20  $\mu$ g) and alum (2 mg). After 1 week, these mice were further sensitized with the same reagent. On day 10 after the second sensitization (day 18), the mice were exposed to aerosols of allergen (1% [wt/vol] ovalbumin [grade 3, Sigma-Aldrich, St Louis, Mo] in PBS, pH 7.4) for 10 minutes on 3 consecutive days (days 18–20). The aerosol exposure was performed in a chamber by using a PARI nebulizer (PARI Japan, Osaka, Japan). As a control group, the mice were sensitized with ovalbumin and exposed to aerosols of PBS.

### Administration of endostatin or anti-VEGF receptor antibodies

Murine Fc-endostatin was generated and kindly provided by National Cancer Institute, BRB Pre-clinical Repository (Frederick,

Md; <http://www.ncifcrf.gov/research/brb/site/preclinrepo.asp>). Endostatin (20 mg/kg,<sup>25</sup> twice a day) was subcutaneously administered on 4 consecutive days from day 18 to day 21. In some mice, control murine Fc protein (a generous gift from Dr David M. Briscoe) was given as control. The specific neutralizing mAbs directed against VEGF receptor (VEGFR)–1 and VEGFR–2; R1 mAb; MF-1 and R2 mAb; DC101, respectively) were provided by ImClone Systems (New York, NY). R1 mAb, R2 mAb, or both combined (1 mg/mouse for each mAb) were administered intraperitoneally every other day from day 17 to day 21.<sup>26</sup> For the control, the same isotype (IgG<sub>1</sub>) raised against mouse IgA (1 mg/100  $\mu$ L in PBS per mouse) was injected every other day from day 17 to day 21 (Pharmingen, San Diego, Calif).<sup>27</sup> Mice administered endostatin, R1 mAb, R2 mAb, or both R1 and R2 mAb combined appeared healthy, showed regular weight gain and activity levels similar to control mice, and had no ulceration at the injection sites.

### Pulmonary function testing

Airway responsiveness of mice to increasing concentrations of aerosolized methacholine was measured by using whole-body plethysmography (Buxco, Wilmington, NC) as previously described.<sup>28</sup> The main indicator of airflow obstruction, enhanced pause (Penh), which shows strong correlation with the airway resistance examined by standard evaluation methods, was calculated from the box waveform.<sup>29</sup> Aerosolized saline or methacholine in increasing concentrations (6, 12, 25, 50 mg/mL) was nebulized to the mice for 1 minute, and Penh measurements were taken for 5 minutes after each dose. Penh values for the 5 minutes were averaged and used to compare results across treatment groups and individual mice.

### Pathological analysis

After physiologic testing in airway-sensitized mice or postallergen-challenged mice, the animals were euthanized with sodium pentobarbital (Nakalai Tesque Inc, Kyoto, Japan). Serum was collected and stored at –20°C. Bronchoalveolar lavage (BAL) was performed, and BAL fluid (BALF) cells and differentials were counted as previously described.<sup>28</sup> After lavage, some of the lungs were instilled with 10% buffered formalin, removed, and fixed in the same solution. After paraffin embedding, sections for microscopy were stained with hematoxylin and eosin (H&E). An index of pathologic changes in coded H&E slides was delivered by scoring the inflammatory cell infiltrates around airways and vessels for greatest severity (0, normal; 1, <3 cell diameters thick; 2, 4–10 cells thick; 3, >10 cells thick) and overall extent (0, normal; 1, <25% of sample; 2, 25% to 50%; 3, 51% to 75%; 4, >75%). The index was calculated by multiplying severity by extent, with a maximum possible score of 9. Metaplastic goblet cells were identified by their abundant cytoplasm with mucin and flattened nuclei. At 48 hours after challenge, lungs were removed from mice for evaluation of the expression of VEGF. The lungs were instilled with 10% buffered formalin, removed, and fixed in the same solution. Immunohistochemical staining of paraffin sections using an anti-VEGF monoclonal antibody (clone JH121; 1:50 dilution; Upstate Biotechnology, Lake Placid, NY) or an isotype control IgG (DAKO Japan, Kyoto, Japan) was performed with the streptavidin-biotin complex method (DAKO Japan). Slides were evaluated light-microscopically. Ovalbumin-sensitized and PBS-exposed mice were used as control.

### Assay of serum IgE and VEGF

Antiovalbumin-specific IgE Ab was measured by ELISA as previously described.<sup>30</sup> For a positive control standard, we used a monoclonal antiovalbumin IgE (2C6; Serotec, Kidlington-Oxford, United Kingdom).<sup>28</sup> The content of VEGF in serum was measured with an

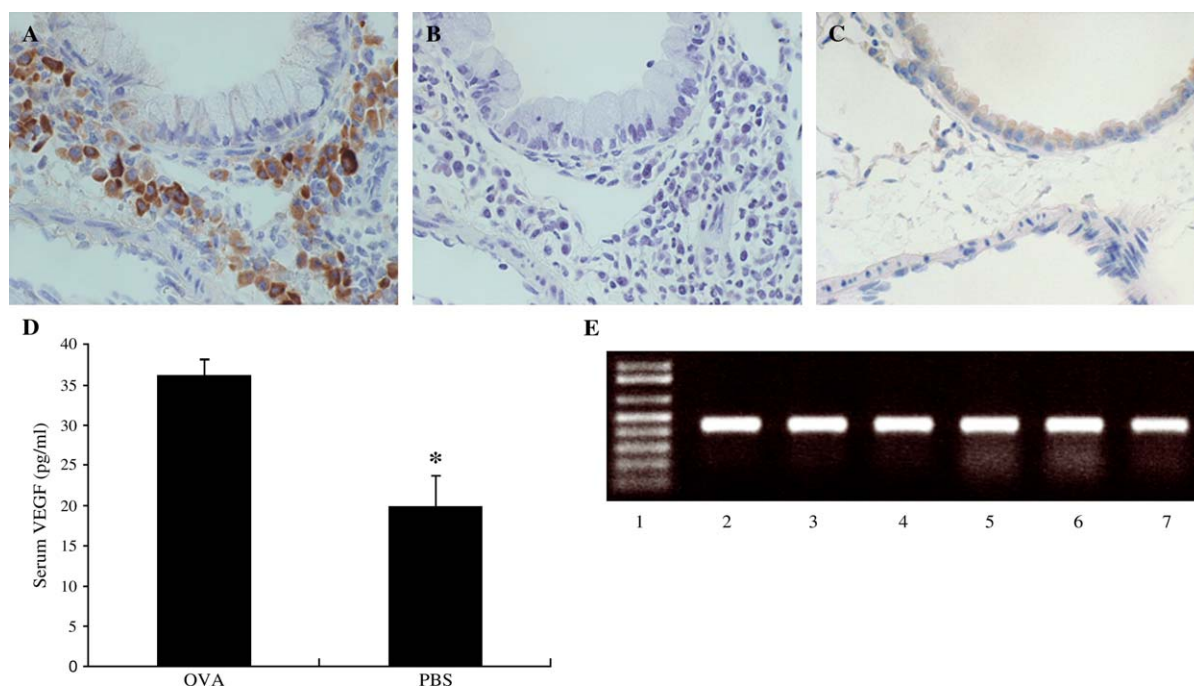


**FIG 1.** VEGF expression in the airways, serum VEGF levels, and neovascularization. Immunohistochemistry of VEGF shows increased expression on inflammatory cells after ovalbumin (OVA) challenge (**A**) compared with isotype control (**B**) or PBS challenge (**C**). **D**, Immunoassay of serum VEGF,  $n = 9$ , each group.  $*P < .02$ . **E**, CD31 mRNA expression measured by RT-PCR. Lanes: 1, size marker; 2-4, PBS-challenged mice; 5-7, OVA-challenged mice.

ELISA kit (R&D Systems Inc, Minneapolis, Minn). The detection limit of the VEGF was 3.0 pg/mL.

### Extraction of total RNAs, RT-PCR, and quantitative real-time PCR analysis

Total RNA was isolated from lung tissue by using guanidine isothiocyanate methods and was transcribed to cDNA by using Omniscript Reverse Transcriptase (QIAGEN, Hilden, Germany) and amplified with oligo dT primers (Amersham Biosciences Corp, Piscataway, NJ). PCR was performed by thermal cycler for 35 cycles of 94°C for 30 seconds, 58°C for 45 seconds, and 72°C for 45 seconds, and final extension was performed at 72°C for 10 minutes. The PCR products were examined by 2.5% agarose gel electrophoresis. To evaluate neovascularization, we performed semiquantitative RT-PCR analysis to examine the mRNA expression of CD31 as previously described.<sup>31</sup> The primer for mouse CD31 was designed from the published sequence (sense: 5'-TGCTGTTCTATAAG-GACGAT-3' [285-304]; antisense: 5'-CACTCCTGATGGGTTCT-GACAG-3' [719-738]). PCR was performed at 94°C for 1 minute, at 53.5°C for 1 minute, and at 72°C for 1 minute for 30 cycles. Several cytokines, chemokines, and adhesion molecule expressions in the lungs were analyzed by quantitative real-time RT-PCR. Real-time PCR analysis was performed by using ABI Prism 7700 sequence detector system (PE Applied Biosystems, Foster City, Calif). Primer/probe sets were purchased from PE Applied Biosystems. PCR was performed with the TaqMan Universal PCR Master Mix (PE Applied Biosystems) by using 1  $\mu$ L cDNA in a 20  $\mu$ L final reaction volume. The PCR thermal cycle conditions were as follows: initial step at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The expression levels of each

mRNA were divided by levels of mRNA of the housekeeping gene  $\beta$ 2-microglobulin; mRNA levels within the ovalbumin-sensitized and PBS-exposed mice were assigned an arbitrary value of 1.

### Statistical analysis

Data are presented as means  $\pm$  SEs. ANOVA analysis of differences among group means was performed by using the Fisher protected least significant difference test and the Statview software program (Abacus Concepts, Berkeley, Calif). Statistical significance was accepted when  $P < .05$ .

## RESULTS

### Increased VEGF-positive cells in the airways and VEGF levels in serum after allergen challenge

Immunohistochemical analyses showed increased immunoreactive VEGF within inflammatory cells such as eosinophils and macrophages around the airways of ovalbumin-sensitized and exposed mice compared with mice sensitized with ovalbumin and exposed to PBS (Fig 1, A-C; the data are representative of 5 histologic samples from 5 mice). Furthermore, enzyme immunoassay revealed that serum VEGF levels were significantly increased in ovalbumin-exposed mice compared with mice exposed to PBS (Fig 1, D). On the other hand, the evaluation of neovascularization by RT-PCR for CD31

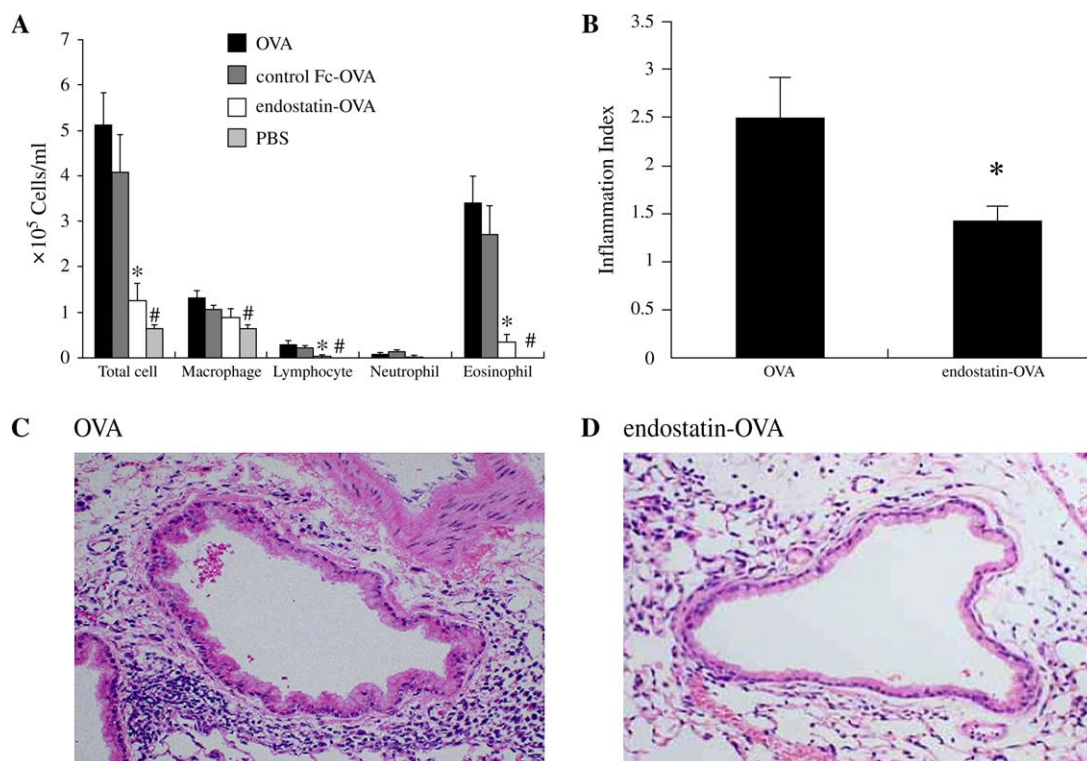


**FIG 2.** Effect of endostatin on allergic pulmonary inflammation. **A**, BAL cell findings.  $n > 10$  for each group.  $*P < .03$ ;  $\#P < .02$  versus ovalbumin (OVA) group and OVA group with control Fc-protein treatment. **B**, Semiquantitative analysis of histopathologic changes,  $n = 5$  per group.  $*P < .03$ . **C and D**, Representative lung histology findings from OVA and OVA with endostatin treatment groups. Original magnifications,  $\times 200$ .

expression in airways in this acute asthma model showed no significant increases over control samples from ovalbumin-sensitized and PBS-exposed mice (Fig 1, E).

### Effects of endostatin treatment

Next we evaluated the effect of endostatin administration on key features of our mouse model of asthma. Ovalbumin-sensitized and exposed mice showed increased eosinophils on BALF compared with control mice sensitized with ovalbumin and exposed to PBS (Fig 2, A). Endostatin treatment significantly decreased the number of total cells, lymphocytes, and eosinophils on BALF (Fig 2, A). Results of semiquantitative scoring of histological changes further support the qualitative changes presented in Fig 2, B. Although control mice showed robust pathologic changes of allergic pulmonary inflammation (AI) (eosinophil and mononuclear cell infiltration around airway and vessels and goblet cell hyperplasia), endostatin treatment diminished the pathologic changes of AI (Fig 2, C and D). Airway hyperresponsiveness (AHR) was assessed as an increased Penh in response to increasing doses of methacholine. Ovalbumin-sensitized and exposed mice showed AHR (Fig 3), manifest as increased Penh values compared with those seen in control mice sensitized with ovalbumin and challenged with aerosols of PBS. In contrast, ovalbumin-sensitized and exposed mice treated with endostatin showed markedly decreased AHR, comparable to the control group

(Fig 3). Serum ovalbumin-specific IgE was increased in ovalbumin-sensitized and exposed mice. Endostatin treatment markedly reduced serum ovalbumin-specific IgE production (Fig 4). In contrast to endostatin, control murine Fc protein administration did not show any effects on key features of our mouse model of asthma.

### Role of VEGF

Because actions of endostatin may be linked to antagonism of VEGF,<sup>32</sup> we investigated whether the dramatic effects seen with endostatin could be reproduced by blockade of the VEGF pathway. We treated animals with neutralizing monoclonal antibodies of VEGFR-1 or VEGFR-2 (R1 mAb, R2 mAb, or both combined). The data in Fig 5, A, show significant decreases in AI (decreased total cells, lymphocytes, and eosinophils on BALF) in R2 mAb-treated ovalbumin-sensitized and challenged mice. In contrast, R2 mAb treatments did not affect the AHR or prevent increases in serum ovalbumin-specific IgE (Fig 5, B and C). When we treated mice with R1 mAb or both R1 mAb and R2 mAb combined, we obtained results similar to those when we used R2 mAb alone (Table I).

### Endostatin downregulates inflammatory mediators

To characterize further the underlying mechanisms responsible for the marked decrease in allergic response

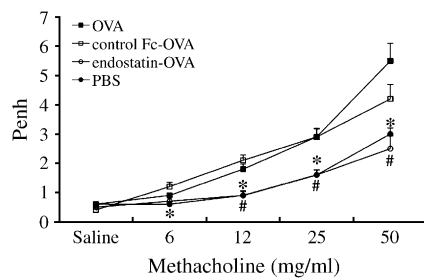


**FIG 3.** Effect of endostatin on airway responsiveness to inhaled methacholine, measured by whole-body plethysmography.  $n = 6$  for each group. \* $P < .03$ ; # $P < .05$  versus ovalbumin (OVA) group and OVA group with control Fc protein treatment.

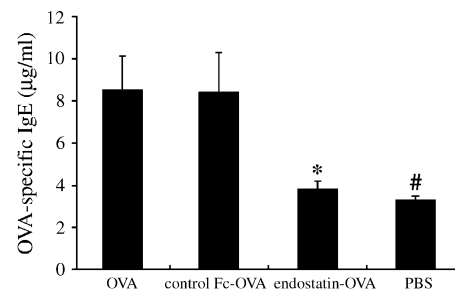


**FIG 4.** Effect of endostatin on the production of serum ovalbumin (OVA)-specific IgE.  $n = 10$  for each group. \* $P < .01$ ; # $P < .03$  versus OVA group and OVA group with control Fc protein treatment.

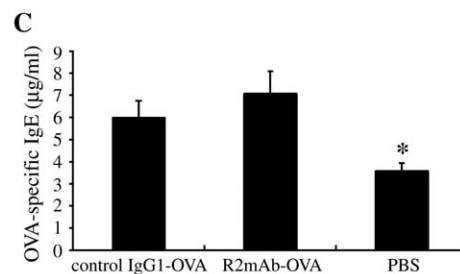
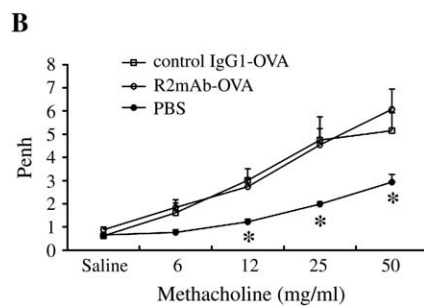
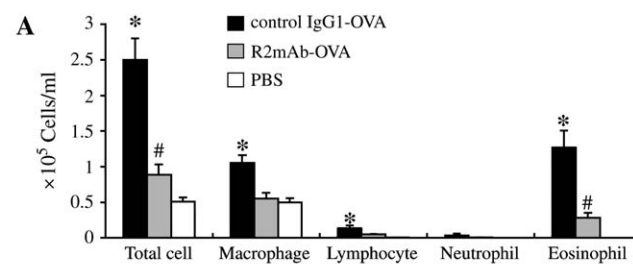


**FIG 5.** Effect of neutralizing antibodies to VEGFR-2 (R2 mAb). **A**, BAL cells.  $n > 9$  per group. \* $P < .02$  versus other groups; # $P < .04$  versus PBS group. **B**, Airway responsiveness to inhaled methacholine, measured by whole-body plethysmography.  $n = 5$  per group. \* $P < .03$  versus other groups. **C**, Analysis of serum allergen-specific IgE.  $n = 6$  per group. \* $P < .03$  versus other groups.

**TABLE I.** Effect of neutralizing monoclonal antibodies of VEGFR-1 (R1 mAb) on pathophysiological features of asthma†

|                                       | Control IgG <sub>1</sub> – OVA | R1mAb – OVA     | R1mAb + R2mAb – OVA | PBS                |
|---------------------------------------|--------------------------------|-----------------|---------------------|--------------------|
| BAL                                   |                                |                 |                     |                    |
| Total cells ( $\times 10^5$ cells/mL) | 5.73 $\pm$ 0.53#               | 3.06 $\pm$ 0.51 | 2.71 $\pm$ 0.43     | 0.4 $\pm$ 0.02*    |
| Eosinophils ( $\times 10^5$ cells/mL) | 4.01 $\pm$ 0.47#               | 1.46 $\pm$ 0.28 | 1.71 $\pm$ 0.37     | 0.002 $\pm$ 0.001* |
| OVA-specific IgE ( $\mu$ g/mL)        | 8.90 $\pm$ 1.55                | 8.71 $\pm$ 1.22 | 8.94 $\pm$ 1.85     | 2.73 $\pm$ 0.34*   |
| Airway physiology (Penh)              |                                |                 |                     |                    |
| Methacholine 12 mg/mL                 | 1.47 $\pm$ 0.15                | 2.59 $\pm$ 1.01 | 1.45 $\pm$ 0.25     | 0.86 $\pm$ 0.14*   |
| Methacholine 25 mg/mL                 | 4.55 $\pm$ 0.80                | 4.98 $\pm$ 1.87 | 5.77 $\pm$ 0.89     | 1.56 $\pm$ 0.18*   |
| Methacholine 50 mg/mL                 | 11.7 $\pm$ 3.30                | 9.77 $\pm$ 2.65 | 10.88 $\pm$ 2.85    | 3.04 $\pm$ 0.20*   |

\* $P < .05$  versus 3 other groups.

† $n = 5$  for each group.

# $P < .02$  versus ovalbumin (OVA) group treated with R1 mAb and both R1 mAb and R2 mAb combined.

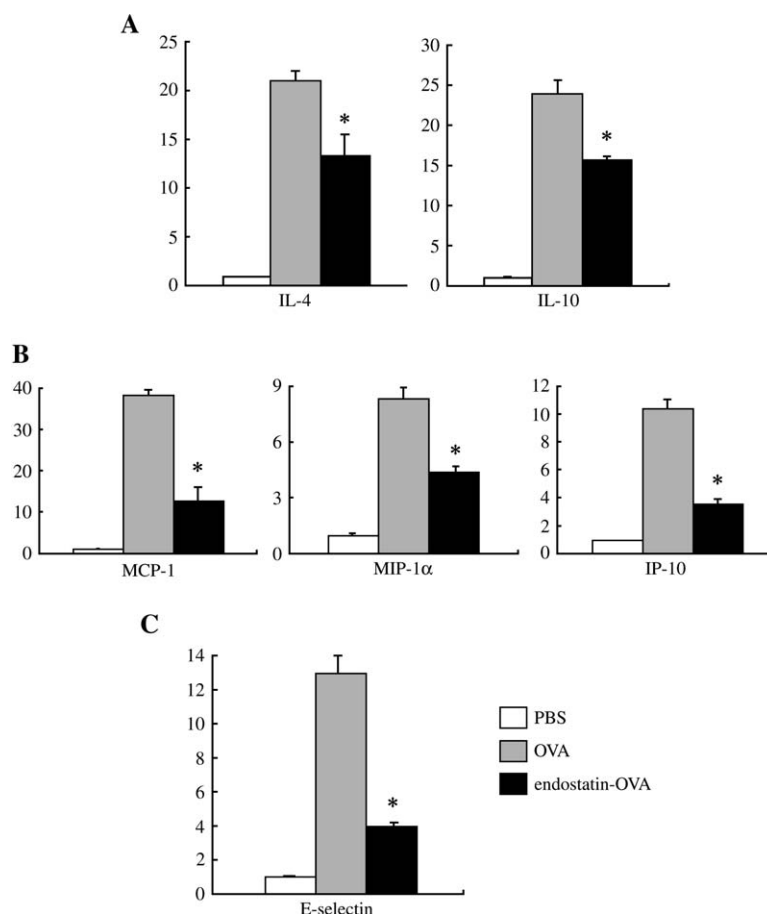


**FIG 6.** Effect of endostatin on mRNA expression of immune mediators in the lung measured by quantitative real-time PCR. Lungs were collected from PBS, ovalbumin (OVA), and endostatin-treated OVA group. Results are normalized to  $\beta$ 2-microglobulin expression and are expressed as means  $\pm$  SEMs of triplicate wells. The results are representative of 5 independent experiments. \* $P < .01$  versus the OVA group.

in lungs of endostatin-treated mice, we analyzed local expression of cytokines and chemokines by quantitative real-time PCR. The expressions of IL-4 and IL-10 were significantly upregulated in ovalbumin-sensitized and exposed mice compared with mice sensitized with ovalbumin and exposed to aerosols of PBS (Fig 6, A). Endostatin treatment significantly downregulated these  $T_H2$  cytokines (Fig 6, A). Furthermore, some potent chemokines that are responsible for inflammation in physiological as well as pathological conditions were also suppressed by endostatin treatment. These chemokines include monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ), and interferon-inducible protein 10 (IP-10) (Fig 6, B). In addition, E-selectin, an inducible leukocyte adhesion molecules specifically expressed by endothelial cells, was significantly suppressed by endostatin treatment (Fig 6, C). On the other hand,  $T_H1$  cytokines including TNF- $\alpha$  and IFN- $\gamma$  were not downregulated by endostatin treatment (data not shown). We also examined the expression of mRNAs for matrix metalloproteinases (MMPs) that have been suggested as a target of endostatin. However,

MMP-2 and MMP-9 expression in the lung was not suppressed by endostatin treatment (data not shown).

## DISCUSSION

We used a murine ovalbumin-induced asthma model to test the ability of the antiangiogenic agent endostatin to block the development of the asthma phenotype. The context for these studies includes data suggesting that angiogenesis may be an important component of the chronic inflammation and airway remodeling in asthma. For example, clinical studies show that the number and percentage of vessels in airways taken from subjects with asthma are higher than in control subjects.<sup>11,13,33</sup> Increased vascularity of the bronchial mucosa in patients with asthma is reported to relate closely to the expression of VEGF in the airways.<sup>17</sup> It is also reported that VEGF and endostatin levels in induced sputum and VEGF/endostatin ratio are significantly higher in subjects with asthma than in control subjects.<sup>34</sup> Moreover, 1 recent study identified a role for the angiogenic mediator

VEGF in an acute model of chemical (toluene diisocyanate)-induced asthma.<sup>35</sup> Recently, Lee et al<sup>36</sup> provided the evidence by using lung-targeted VEGF<sub>165</sub> transgenic mice that VEGF cooperated with T<sub>H</sub>2 cells in the development of allergic lung responses. We also observed increased expression of VEGF within the inflammatory infiltrates around asthmatic airways as well as increased levels of VEGF in the serum in our short-term acute exposure model. Hence, we sought to investigate the potential of blockade of angiogenic factors to prevent the development of acute asthma responses in a mouse model.

There were 2 major findings in this study. First, we found that endostatin, a potent naturally occurring anti-angiogenic protein fragment, significantly prevented the pathophysiological features of asthma. This was demonstrated by attenuation of AHR, decreased AI, and diminished production of allergen-specific IgE. This study used noninvasive plethysmography to measure Penh as an index of AHR, a technique that has been the subject of considerable controversy.<sup>37</sup> However, the best, and arguably most useful, correlation of Penh with more invasive measures of airway function is found in BALB/c mice after ovalbumin sensitization and aerosol challenge protocol, as used in our studies. The semiquantitative analysis of tissue inflammation has limitations because it is not a rigorous morphometric technique. Nevertheless, it complements (and is consistent with) the more quantitative analysis of AI by lavage. Moreover, the combined analysis of airway function, inflammation, and IgE responses is internally consistent. Endostatin actions include inhibition of endothelial cell proliferation and migration and induction of endothelial cell apoptosis and cell cycle arrest.<sup>18,19,38,39</sup> More specifically, blockade of VEGF/VEGFR signaling has been reported to be an example of how endostatin causes reduced endothelial cell proliferation and migration.<sup>32</sup> Hence we investigated whether blockade of VEGF/VEGFR signaling was responsible for the antiasthmatic effect of endostatin in our model by using receptor blockade interventions.

The second major finding of the study implicates an important but partial role for VEGF. Blockade of VEGF receptors using the specific neutralizing monoclonal antibodies led to decreased AI, findings similar to those reported in a model of toluene diisocyanate-induced asthma.<sup>35</sup> In contrast with their findings, in our model, VEGF receptor blockade did not affect AHR or increase serum ovalbumin-specific IgE. The basis for this is not clear but may reflect our use of a protein allergen (ovalbumin) model of asthma. It is also worth noting that in spite of increased expression of VEGF within the inflammatory infiltrates around asthmatic airways as well as increased levels of VEGF in the serum, no histological features of airway remodeling and neovascularization are seen in our short-term acute exposure model. Evaluation of vascularity by immunostaining and RT-PCR for CD31 expression in airways in this acute asthma model showed no significant increases over control samples from ovalbumin-sensitized and PBS-exposed mice. Thus, our data

suggest that VEGF may play an important (albeit partial) role early in the development of asthma when histological features of angiogenesis are not seen yet. We speculate that VEGF mainly functions as a proinflammatory cytokine in the incipient stage of asthma.

Our data showing only partial inhibition of asthma features (AI, not AHR and IgE) by VEGF receptor blockade indicate that the effect of endostatin is not simply a result of antagonism of VEGF signaling but may also operate through other mechanisms. Recently, Abdollahi et al<sup>40</sup> provided evidence that endostatin induces its anti-angiogenic effect through regulation or modification of a large cluster of genes known to affect endothelial growth and development using genome-wide expression profiling, coupled with RT-PCR and phosphorylation analysis. Therefore, it is likely that the therapeutic efficacy of endostatin may work through other mechanisms distinct from antagonism of VEGF signaling. Our real-time PCR data indicated the downregulation of several inflammatory mediators including T<sub>H</sub>2 cytokines (IL-4, IL-10), some potent chemokines (MCP-1, MIP-1 $\alpha$ , IP-10), and E-selectin. These mediators are known to be associated with pathogenesis of asthma. Thus, the beneficial effect of endostatin on asthma development may depend, at least in part, on the inhibition of local immune activation, thereby resulting in the prevention of infiltration of immune activating cells into the lung. However, we speculate that the other multiple actions of endostatin might contribute to the dramatic inhibition of physiologic (AHR), pathologic (BAL and histopathology), and serologic (IgE) measures of asthma severity in our model.

In conclusion, we have demonstrated for the first time that the administration of recombinant endostatin can prevent the development of key features of asthma in a mouse model (AHR, AI, increased IgE). Our data indicate that angiogenic factors contribute to the asthma phenotype even before increased vascularity or airway remodeling occurs, probably via proinflammatory or indirect mechanisms. It is noteworthy that endostatin is already in use in human clinical trials for several other disease states. Thus, further studies of the therapeutic potential of endostatin and other antiangiogenic factors for both acute and chronic aspects of asthma are warranted.

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