



Role of vascular endothelial growth factor antagonism on airway remodeling in asthma

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ABSTRACT

Background: Vascular endothelial growth factor (VEGF) is an important mediator of the neoangiogenesis component of remodeling in asthma.

Objective: To evaluate the influence of VEGF blockage on airway remodeling, specifically epithelium thickness, subepithelial smooth muscle thickness, number of mast and goblet cells, and basement membrane thickness, in a mouse model of chronic asthma.

Methods: We used 30 BALB/c mice. The control group was not exposed to ovalbumin or any medication (group 1). Other groups were exposed to intraperitoneal and inhaled ovalbumin to achieve chronic asthma. Each of these groups received intraperitoneal saline (group 2), intraperitoneal dexamethasone (group 3), or intraperitoneal bevacizumab (group 4). Histomorphologic examination for epithelium thickness, subepithelial smooth muscle thickness, number of mast and goblet cells, and basement membrane thickness was performed from the middle zone of the left lung.

Results: Treatment with anti-VEGF caused significant reduction in epithelial, subepithelial muscle, and basement membrane thickness compared with untreated asthmatic mice ($P = .001$, $P = .03$, and $P = .009$, respectively). Goblet and mast cell numbers were significantly lower in mice treated with anti-VEGF than in untreated mice ($P = .02$ and $P = .007$, respectively). Dexamethasone treatment resulted in improvement of all histomorphologic markers, except goblet cell number. Influences of dexamethasone and anti-VEGF on epithelial and basement membrane thickness and mast and goblet cell numbers did not differ ($P > .05$), but subepithelial muscle layer was thinner in the former ($P = .003$).

Conclusion: VEGF blockage may provide adjunctive therapeutic options as steroid-sparing agents for more effective treatment of remodeling in asthma.

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Introduction

Asthma is a chronic inflammatory disease of the airways, which in the long term results in remodeling characterized by inappropriate injury and repair response.^{1,2} Various mediators, such as transforming growth factor β (TGF- β), interleukin (IL) 15, IL-13, metalloproteinases, and angiogenic factors, released from the inflammatory cells in the microenvironment lead to the mucosal and submucosal airways, causing microstructural and

macrostructural changes.¹ These changes include epithelial dis-integrity, reticular basement membrane thickening, mucous gland hyperplasia, smooth muscle and subepithelial fibrosis, and vascular remodeling.³ Vascular changes are major components of chronic inflammation and remodeling in asthma and are complex processes caused by the proangiogenic action of the listed mediators.⁴ Among these proangiogenic mediators, vascular endothelial growth factor (VEGF), released from many different cell types, including macrophages, mast cells, and epithelial cells, is considered the most important and is suggested to play a role in vascular and extravascular remodeling and inflammation and increased vascular permeability.^{1,4–6} Clinical significance of vascular remodeling lies in the fact that it leads to airway wall thickening and narrowing of the lumen.⁴ Moreover, airway wall angiogenesis increases inflammatory mediator delivery to the airway,

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contributing to a vicious cycle of remodeling.⁷ Once remodeling develops, it leads to a decrease in bronchodilator responsiveness and persistent obstruction to airway despite treatment.⁷

Although we have concise knowledge about the presence of remodeling in asthma, there is still a gap in the information regarding its prevention or treatment. Current medications are not sufficient to prevent development of remodeling despite the fact that they provide clinical and symptomatic improvement.³ The most promising agents currently used that have a partial therapeutic role are corticosteroids.⁷ Considering the subsequent clinical effect of airway remodeling in asthma, it is vital to prevent its development. The major barrier to therapeutic advances is the fact that many mediators and inflammatory cells play an active role in pathogenesis.³ Therefore, the aim of this study was to evaluate the influence of VEGF blockage on airway remodeling, specifically epithelium thickness, subepithelial smooth muscle thickness, the number of mast and goblet cells, and basement membrane thickening, in a mouse model of chronic asthma.

Methods

Experimental Animals

We used 6- to 8-week-old BALB/c mice weighing 18 to 20 g for the experiment, and they were kept in hygienic Macrolene cages in air-conditioned rooms on a 12-hour light-dark cycle. Food and water were provided to a total of 30 mice ad libitum throughout the experiment. All experimental procedures complied with the requirements of the Animal Care and Ethics Committee (protocol 54/2010).

Sensitization and Inhalation Exposure

We used the chronic murine model of asthma described by Temelovski et al.⁸ BALB/c mice have high IgE response to ovalbumin, and we sensitized these mice via intraperitoneal administration of 10 μ g of chicken egg ovalbumin (grade V; Sigma, St Louis, Missouri) twice 14 days apart (21 and 7 days before ovalbumin inhalation).

Mice sensitized with intraperitoneal chicken egg ovalbumin were exposed to 2.5% aerosolized ovalbumin in sterile saline for 30 minutes per day on 3 days every week up to 8 weeks beginning on the 21st day of the experiment. A whole-body inhalation system was used for the inhalation procedure during which mice were placed in chambers sized 41 $\times$ 26 $\times$ 21 cm connected to a jet nebulizer. A total of 1% ovalbumin solution in normal saline will be aerosolized via delivery of compressed air to the jet nebulizer and injection to the connecting tube. Aerosol delivered via this nebulizer system achieves a diameter of 4 μ m or less in 80% or more of the particles. Particle concentration of 10 to 20 mg/m³ was maintained. The same system was used to deliver saline inhalation to mice in the control group.

Study Design

Mice enrolled in the study were grouped into 4 groups of 7 mice each. Group 1, the control group, contained mice not exposed to intraperitoneal or inhaled ovalbumin or any other medication. The other 3 groups were exposed to intraperitoneal and inhaled ovalbumin to achieve chronic asthma. Among these 3 groups, one received a dose of intraperitoneal saline per week for 2 weeks (group 2), whereas the other group was injected with 1 mg/kg of dexamethasone intraperitoneally for 7 days during the last week of ovalbumin inhalation (group 3). The last group of mice received a dose of intraperitoneal bevacizumab, 0.15 mg intraperitoneally (5 mg/kg) per week for 2 weeks (group 4) (Fig 1).

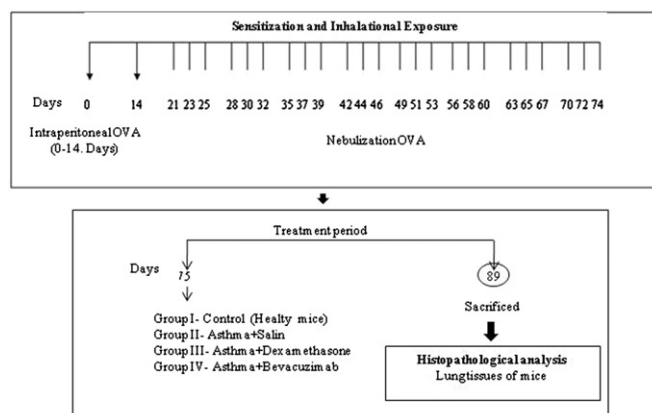


Figure 1. Sensitization and inhalation exposure and treatment groups.

Histomorphologic Evaluation

Samples obtained from the middle zone of the left lung of mice were placed in buffered formalin for light microscopic evaluation while a 1-mm³ sample was placed in Karnovsky fixative for electron microscopic evaluation.

The dissected tissue samples were fixed in 10% formalin in phosphate buffer, pH 7.4, overnight, then dehydrated through a series of increasing concentration of ethanol (60%, 70%, 80%, 90%, and absolute ethanol) and Xylene and then embedded in paraffin blocks. Paraffin blocks were placed in a Leica RM2125 rotary microtome (Leica, Solms, Germany), and sections of 5- μ m thickness were obtained. Sections were deparaffinized, hydrated, and stained with hematoxylin-eosin, Toluidine blue, and periodic acid–Schiff. For light microscopic evaluation, airway inflammation, epithelium, subepithelial smooth muscle thickness, mucin-secreting goblet cells, and mast cells were examined. Photomicrographs were taken with an Olympus DP25 (Japan) camera, which was adapted on an Olympus CX-41 model microscope (Olympus Optical, Tokyo, Japan). The histologic analysis was performed with a computer-assisted image analyzer system (DP2-BSW).

For ultrastructural investigations, the tissues were postfixed with osmium tetroxide, dehydrated in a graded series of alcohol, and then embedded in Araldite CY212. The thin (60–90 nm) sections were obtained with ultramicrotome (Leica) and stained with uranyl acetate and lead citrate, examined on a transmission electron microscope (Carl Zeiss Libra 120 EFTEM; Carl Zeiss, Jena, Germany), and digitally photographed.

Statistical Analysis

Statistical analysis was performed using SPSS statistical software, version 15.0 (SPSS Inc, Chicago, Illinois). The Kruskal-Wallis nonparametric test was used to compare the results among all 4 groups, whereas the Mann-Whitney *U* test was used to compare the groups in two. *P* < .05 was regarded as statistically significant.

Results

Comparison of All Groups

Mast cells counts per 20,000 μ m² were significantly different among the groups, with the lowest median number of mast cells in group 1 (median, 0.36 per 20,000 μ m²; *P* < .001). On the other hand, the highest number of mast cells was observed in group 2 (median, 1.25 per 20,000 μ m²). The median goblet cell counts per 100 μ m were significantly different among the groups (*P* = .001). The lowest and highest numbers of goblet cells were observed in groups 1 and 2, with median values of 0.50 and 1.62, respectively. Epithelial

thickness and subepithelial muscle thickness were significantly different among the groups ($P = .001$ and $P < .001$, respectively). Similarly, basement membrane thickness was significantly different among the groups ($P < .001$), and group 2 had the highest median value with $682.3 \mu\text{m}$ (Table 1 and Fig 2). All histomorphologic parameters, including mast cell and goblet cell number and epithelial, subepithelial muscle, and basement membrane thickness, were significantly higher in the untreated asthma group compared with the nonasthmatic group ($P = .002$ for all).

Epithelial Thickness

Epithelial layer thickness was not significantly different between group 1 and groups 2 and 3 ($P = .52$ and $P = .85$, respectively). Similarly, epithelial thickness were not significantly different between groups 3 and 4 ($P = .35$). On the other hand, groups 3 and 4 had epithelial thickness significantly lower than group 2, with median values of 13.6 and $14.3 \mu\text{m}$ vs $23.7 \mu\text{m}$, respectively ($P = .001$ for both).

Smooth Muscle Thickness

Subepithelial muscle layer thickness was significantly higher in group 1 compared with group 3 but not significantly different from group 4 ($P = .03$ and $P = .22$, respectively). Both groups 3 and 4 had significantly thinner subepithelial muscle layers compared with group 2 ($P = .001$ and $P = .03$, respectively). Moreover, group 3 had significantly thinner subepithelial muscle layer compared with group 4 (4.6 vs $6.3 \mu\text{m}$, respectively; $P = .003$) (Table 1 and Fig 3).

Basement Membrane Thickness

Basement membrane thickness was significantly lower in group 1 compared with groups 3 and 4 ($P = .002$ and $P = .003$, respectively). Basement membrane thickness in groups 3 and 4 was significantly lower than that in group 2 (503.6 and $540.4 \mu\text{m}$ vs $682.3 \mu\text{m}$; $P = .001$ and $P = .009$, respectively). However, basement membrane thickness was not significantly different between groups 3 and 4 ($P = .35$ and $.53$, respectively) (Table 1 and Fig 4).

Mast Cell Numbers

Mast cells were significantly lower in group 1 compared with groups 3 and 4 ($P = .003$ and $P = .01$, respectively). The median number of mast cells in group 2 was higher than in group 4 but not significantly different from group 3 ($P = .007$ and $P = .07$, respectively). The median number of mast cells was not significantly different between groups 3 and 4 ($P = .16$).

Goblet Cell Numbers

The median numbers of goblet cells in groups 3 and 4 were significantly lower than in group 2 (both $P = .02$). On the other hand, the median number of goblet cells in group 1 was not significantly different than in group 4 ($P = .25$). Similarly, the median number of mast cells was not significantly different between groups 3 and 4 ($P = .21$).

Discussion

This study found that, in a mouse model, untreated chronic asthma is associated with higher mast cell and goblet cell numbers and thicker epithelium, subepithelial muscle layer, and basement membrane. Moreover, both steroid and anti-VEGF treatments are associated with lower mast cell and thinner epithelial, subepithelial muscle, and basement membrane layers. However, steroid treatment does not lead to a significantly lower goblet cell number compared with untreated asthmatic patients, whereas anti-VEGF treatment does. Finally, comparison of steroid and anti-VEGF treatment did not reveal a significant difference in histopathologic parameters except for subepithelial muscle thickness, which was thinner in the steroid-treated group.

Remodeling in asthma describes the whole group of changes in the airway cellular and molecular components.⁹ Histologically remodeling in asthma is characterized by epithelium injury, hyperplasia of goblet cells, thickening in the basement membrane and airway smooth muscle layer, and angiogenesis and increased extracellular matrix.^{9,10} Inflammation and remodeling are thought to be interdependent.¹⁰ In concordance with this, our results demonstrated thickened epithelium, subepithelial smooth muscle, and basement membrane and increased mast and goblet cell number in untreated mice with chronic asthma compared with nonasthmatic mice.

Mast cells, as the primary cells of inflammation, are the major source of angiogenic mediators in chronic inflammation and eosinophils and macrophages.⁴ Therefore, mast cells are not only mediators of immediate and long-term inflammation but also a mediator of remodeling.¹¹ Previous research has demonstrated that mast cell numbers in airways are correlated with disease severity.¹¹ Decrease in the number of mast cells was achieved with both steroid and anti-VEGF treatment, which might have contributed to the decrease in remodeling observed with both treatments. Moreover, mast cells are involved in the goblet cell hyperplasia indicated in remodeling.¹¹ In this respect, a lower number of goblet cells was observed with a lower number of mast cells in our mice treated with steroid and anti-VEGF compared with the non-treated group. Therefore, the decrease in mast cell number might have contributed to the decrease in goblet cell number.

Steroids are the mainstay of anti-inflammatory treatment in asthma, and many effects on remodeling have been proposed. One of these effects is the antiproliferative effect on fibroblasts, which are known to have a role in remodeling.¹² Moreover, high-dose inhaled steroid treatment leads to a decrease in basement membrane thickness in people with asthma.¹³ Previous research demonstrated that steroid and long-acting bronchodilator treatment leads to a decrease in expression of both VEGF and VEGF receptor in patients with asthma, and this finding correlated with airway remodeling and airway hyperreactivity in patients with asthma.⁶ Our results supported these findings in that asthmatic mice treated with systemic steroids had significantly lower basement membrane thickness compared with untreated asthmatic mice. In addition, when used with long-acting bronchodilators, steroids decrease angiogenic and profibrotic growth factor release in virus-induced asthma.¹⁴ On the other hand, the effects of inhaled

Table 1
Medians (interquartile ranges) of histopathologic markers in the 4 study groups

Marker	Group 1	Group 2	Group 3	Group 4
Epithelium thickness, μm	14.4 (11.9–15.0)	21.4 (19.8–27.8)	13.5 (13.0–14.2)	14.3 (12.9–15.4)
Subepithelial smooth muscle thickness, μm	5.9 (5.0–6.2)	8.3 (6.9–9.9)	4.6 (3.8–4.9)	6.3 (5.7–8.1)
No. of mast cells per 20,000 μm^2	0.4 (0.0–0.4)	1.3 (0.9–1.4)	0.7 (0.7–1.1)	0.7 (0.4–0.7)
No. of goblet cells per 100 μm	0.5 (0.0–0.8)	1.6 (1.6–2.6)	1.4 (1.2–1.5)	0.7 (0.5–1.6)
Basement membrane thickness, nm	311.8 (292.3–364.9)	682.3 (655.9–710.9)	503.6 (430.1–553.2)	540.4 (424.3–628.5)

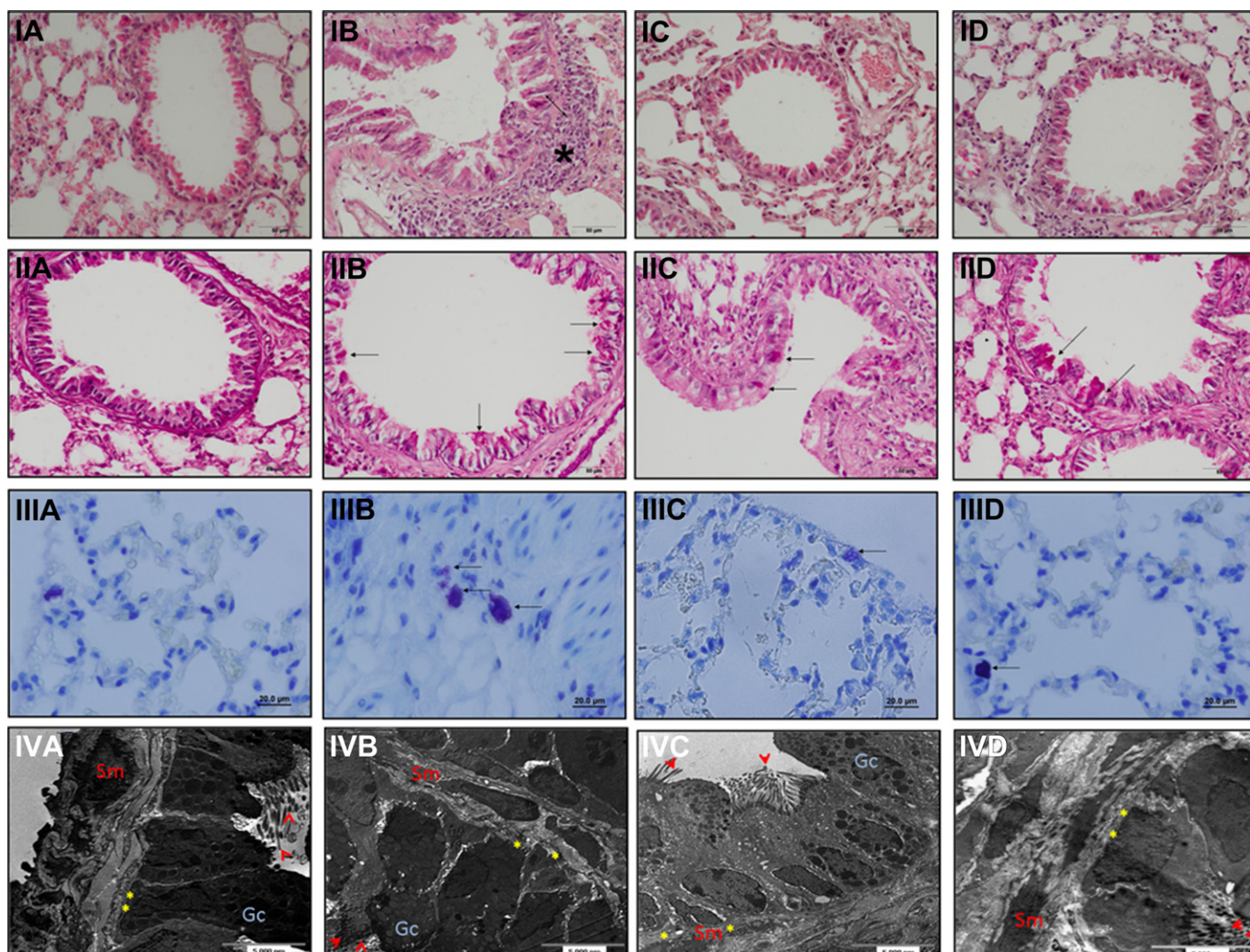


Figure 2. Comparison of the stains in all study groups. I, Hematoxylin–eosin stains for the control group (A), the untreated asthma group (B; double arrow indicates sub-epithelial smooth muscle; asterisk, peribronchiolar infiltration), the dexamethasone group (C), and the anti-VEGF group (D). II, Periodic acid–Schiff stains for the control group (A), the untreated asthma group (B; arrows indicate goblet cells), the dexamethasone group (C; arrows indicate goblet cells), and the anti-VEGF group (D; arrows indicate goblet cells). III, Toluidine blue stains for the control group (A), the untreated asthma group (B; IIIB–C–D; arrows indicate mast cells), the dexamethasone group (C; arrows indicate mast cells), and the anti-VEGF group (D; arrows indicate mast cells). IV, Transmission electron microscopy for the control group (A), the untreated asthma group (B), the dexamethasone group (C), and the anti-VEGF group (D) (Sm indicates smooth muscle; Gc, goblet cell; yellow asterisk, basal membrane; and red arrows, cilia).

steroids on remodeling parameters, such as goblet cell hyperplasia and airway wall thickening, were shown to be dose dependent, and high doses may be associated with systemic effects.¹⁵ Therefore, novel therapeutic agents that act synergistically with steroids on airway remodeling may be required.

VEGF is the main regulator of endothelial cell growth secreted by many different cell types, such as alveolar epithelial cells, fibroblasts, and macrophages.^{10,16} There are 3 receptors of VEGF: VEGF-R1 (Flt-1), VEGF-R2 (Flk-2), and VEGF-R3. VEGF-R2 is thought to play a major role in mitogenic and angiogenic effects.^{17,18} VEGF is an important physiologic mediator of prenatal and postnatal lung growth, but its levels are increased in airways of asthmatic patients, and it plays a major role in pathologic vascular changes in the bronchial wall, extracellular matrix protein release, and induction of inflammation.^{10,16,19} Moreover, VEGF has been demonstrated to be upregulated in many tumors, including breast, lung and gastrointestinal tumors, and angiogenesis has been a major target for antitumor therapy.^{17,18} Bevacizumab is a humanized anti-VEGF monoclonal antibody that prevents activation of VEGF-R, and it was found to inhibit tumor growth.¹⁸ The drug has been used in some cancer types in humans, including colorectal cancer, and it has been reported to be well tolerated despite some serious adverse

effects, such as venous thromboembolism, hypertension, epistaxis, and colorectal ulceration.^{17,20}

The number of VEGF-positive cells in the airway mucosa has been demonstrated to be related to basement membrane thickness.²¹ Moreover, a previous study found that airway smooth muscle cells under strain lead to angiogenesis via secretion of VEGF and that blocking VEGF decreased these angiogenic changes.²² Similarly, another study demonstrated that VEGF secreted from airway smooth muscle cells of asthmatic patients leads to angiogenesis.²³ Blockage of VEGF leads to blockage of angiogenesis, suggesting a role of smooth muscle–originated VEGF in angiogenesis.²³ VEGF secreted by airway smooth muscle is thought to play a role in fibronectin secretion and extracellular matrix modulation, as well as smooth muscle hypertrophy and therefore remodeling.^{16,24} In addition, airway hyperreactivity mostly mediated by airway smooth muscle has been found to decrease with VEGF blockage in a murine model of asthma.²⁵ Not only angiogenesis but also vascular permeability is influenced by VEGF as demonstrated in sputum of asthmatic people, and an increase in vascular permeability, leading to airway edema, is associated with high VEGF levels.²⁶ All these effects of VEGF, including airway edema, angiogenesis, and smooth muscle hypertrophy, contribute

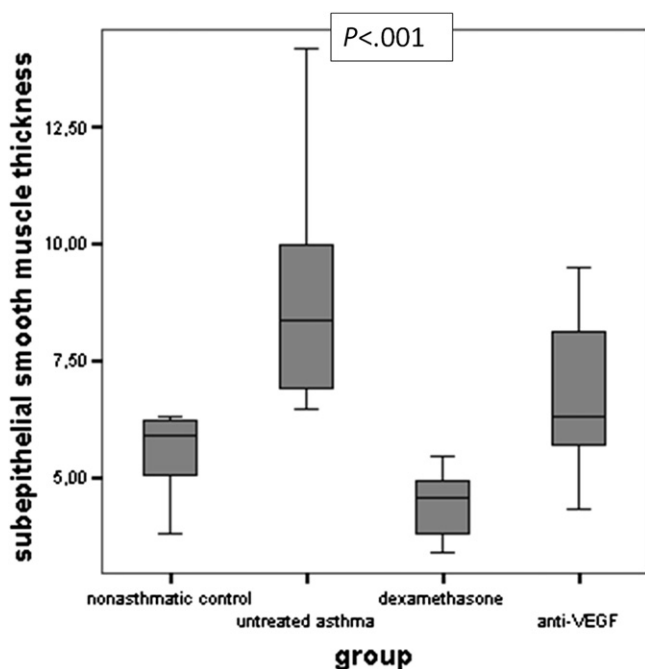


Figure 3. Subepithelial muscle layer thickness among the 4 groups.

to airway lumen narrowing. Thus, in support of our results, blockage of VEGF would be expected to attenuate remodeling due to inhibition of angiogenesis, which will lead to a decrease in thickness of smooth muscle, basement membrane, and epithelium. Some of these effects of VEGF blockage have reported to be related to a decrease in TGF- β 1 levels; however, we have not tested for TGF- β 1 in our study.²⁵

Similar treatment results with both steroid and anti-VEGF in our study might be explained by the fact that inhaled corticosteroids interfere with angiogenesis, and this effect is thought to be mediated mainly by reduced VEGF expression.⁴ Inhaled steroid treatment has been found to lead to a decrease in VEGF-positive cell

number and basement membrane thickness.²¹ Moreover, previous research has demonstrated that 3 months of inhaled steroid treatment leads to decreased VEGF and vessel staining in human bronchial biopsy specimens, indicating that steroid influence on remodeling may be mediated by decreased VEGF activity.²⁷ In addition, VEGF secretion induced by inflammatory cytokines, such as IL-4, IL-5, and IL-13, has been found to be inhibited by exogenous steroids.²⁸ However, in humans it has been demonstrated that even after steroid treatment, sputum levels of VEGF are correlated with airway vascular permeability suggesting that blocking VEGF provides an additive benefit to the clinical and histopathologic improvement observed with steroid treatment.²⁶

In conclusion, systemic treatment with both steroids and VEGF blockers in asthma leads to a decrease in many histomorphologic aspects of remodeling. Inhaled and systemic steroids are already the mainstay of asthma treatment; however, concerns about adverse effects are still a major issue. Therefore, development of safer or inhaled VEGF antagonists may provide adjunctive therapeutic options as steroid-sparing agents for more effective treatment of remodeling.

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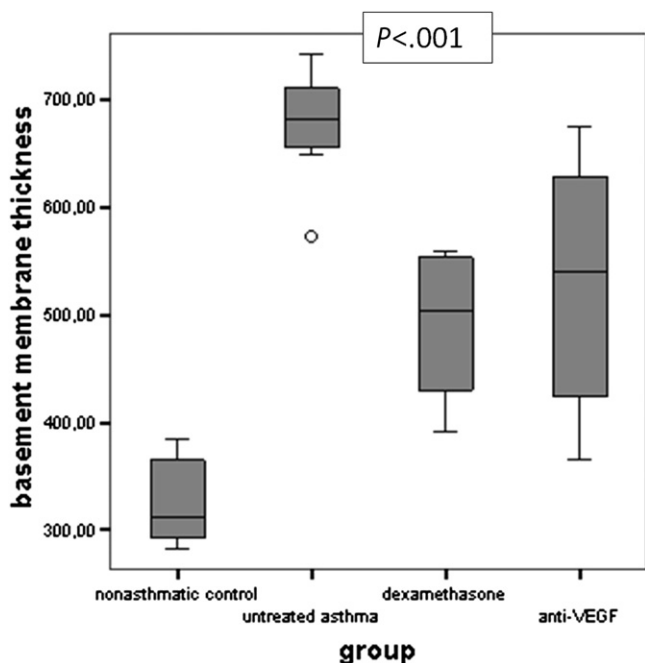


Figure 4. Box plot of basement membrane thickness among the 4 groups.

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