

Gene expression of vascular endothelial growth factor and its receptors and angiogenesis in bronchial asthma

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Background: Angiogenesis is a feature of airway remodeling in bronchial asthma. The mechanism responsible for this angiogenesis is unknown. Vascular endothelial growth factor (VEGF) is a potent inducer of endothelial cells, which may contribute to chronic inflammation and angiogenesis.

Objective: We sought to investigate the molecular mechanisms underlying increased vascularity, and we examined the mRNA expression of VEGF and its receptors (flt-1 and flk-1) within bronchial biopsy specimens from asthmatic patients and normal control subjects.

Methods: Endobronchial biopsy specimens were examined immunocytochemically by staining with anti-type IV collagen mAb to evaluate vessel density by using computer-assisted image analysis. Specimens were also analyzed for the presence of the mRNAs of VEGF and its receptors with in situ hybridization.

Results: The extent of airway vascularity was increased in asthmatic subjects compared with that in control subjects ($P < .01$). Asthmatic subjects exhibited a greater expression of VEGF, flt-1, and flk-1 mRNA⁺ cells in the airway mucosa compared with that in control subjects ($P < .001$ for each comparison). The degree of vascularity was associated with the number of VEGF, flt-1, and flk-1 mRNA⁺ cells. Numbers of cells expressing VEGF mRNA inversely correlated with airway caliber ($r = -0.83$, $P < .01$) and airway hyperresponsiveness ($r = -0.97$, $P < .001$). Colocalization studies showed that macrophages, eosinophils, and CD34⁺ cells were the major sources of VEGF; CD34⁺ cells, macrophages, and T cells expressed both flt-1 and flk-1.

Conclusion: These findings provide evidence that VEGF may play an important role in angiogenesis and subsequent airway remodeling in bronchial asthma. (*J Allergy Clin Immunol* 2001;107:1034-8.)

Key words: Angiogenesis, airway vascularity, vascular endothelial growth factor, flt-1, flk-1, airway remodeling

Asthma is a chronic inflammatory disease associated with airway wall remodeling. Angiogenesis, the growth and proliferation of new blood vessels, is important in a variety of pathophysiologic processes, such as wound

Abbreviations used

Dmin: Minimum cumulative dose

SSC: Standard saline citrate

VEGF: Vascular endothelial growth factor

repair, tumor growth, and rheumatoid arthritis.¹⁻³ Although angiogenesis has not been studied in asthma, an increase in the number of vessels was reported.^{4,5}

Vascular endothelial growth factor (VEGF) is a potent inducer of endothelial cell growth.^{3,6} VEGF was purified on the basis of its ability to induce transient vascular leakage and endothelial cell migration. Studies indicate that VEGF binds to high-affinity cell surface receptors, flt-1 and KDR/flk-1, which are predominantly expressed in endothelial cells.^{7,8} Because angiogenesis and increased vascular permeability are characteristic features of wound healing,⁹ VEGF may play an important role in airway remodeling in asthma.

The aims of this study were to examine the expression of VEGF mRNA and the mRNAs of its 2 receptors within the airways of subjects with mild-to-moderate asthma compared with that of normal control subjects. Furthermore, we investigated the association between VEGF mRNA expression and the extent of vascularity and lung function by using a combination of immunohistochemical and molecular biologic techniques.

METHODS

Study patients

Eleven atopic asthmatic patients were diagnosed according to the criteria of the American Thoracic Society.¹⁰ All patients had typical clinical symptoms, a documented 20% reversibility in peak expiratory flow or FEV₁, and airway hyperresponsiveness. Atopy was defined by positive skin prick test responses to one or more common aeroallergens (ie, mixed grass, tree pollen, mixed molds, cat and dog dander, and house dust mite). Eight nonatopic normal subjects were recruited from the hospital outpatient department. None of the patients were receiving inhaled or oral corticosteroid therapy in the 3 months before the study; for bronchodilation, these patients required only an inhaled β_2 -agonist. All the subjects were nonsmokers, had no evidence of any other pulmonary disease, and had not experienced any upper respiratory tract infections during the 2 weeks preceding the study. The study was approved by the Ethics Committee of the Toho University School of Medicine, and all subjects gave written informed consent. The clinical characteristics of the subjects are given in Table I.

Spirometry and airway methacholine responsiveness was measured

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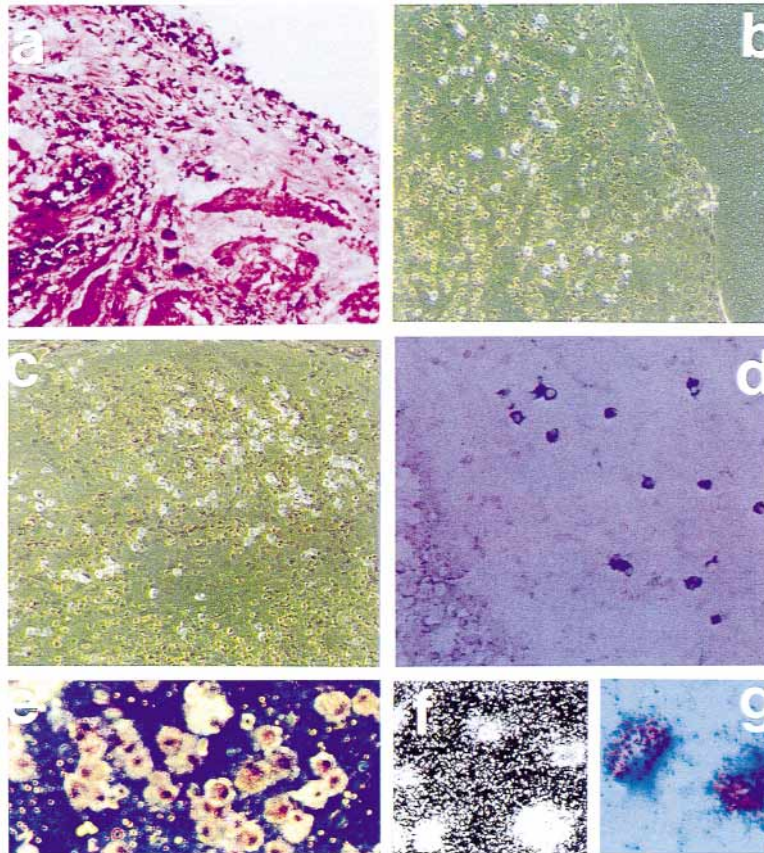


FIG 1. **a**, Hematoxylin and eosin staining of bronchial biopsy specimens from asthmatic subjects. **b**, In situ hybridization of VEGF mRNA by using complementary RNA probe (sulfur 35-labeled) visualized by autoradiography and dark-field illumination in bronchial biopsy specimens from asthmatic subjects. **c**, Dark-field illumination of in situ hybridization for flt-1 in asthmatic airways. **d**, Bright-field illumination of flt-1 in bronchial biopsy specimens from asthmatic subjects. **e**, Simultaneous in situ hybridization and immunocytochemistry in bronchial biopsy specimens from asthmatic subjects showing colocalization of VEGF and CD68. **f**, In situ hybridization of VEGF dark field in asthmatic lungs. **g**, Double staining of major basic protein and VEGF mRNA.

TABLE I. Clinical characteristics of asthmatic and normal control subjects

	Asthmatic subjects (n = 11)	Control subjects (n = 8)
Age (y)	28 (18-39)	33 (24-40)
Sex (M:F)	6:5	4:4
FEV ₁ (% predicted)	67.4 (59.4-83.3)	96.4 (90.2-105.0)
Dmin (units)	0.97 (0.12-3.52)	>50
Skin test response	Positive	Negative

Values are expressed as means and ranges where applicable. A Dmin unit is equal to 1 minute of 1.0 mg/mL aerosol inhalation of methacholine.

with an Astograph (TCK-6000CV; Chest MI Co, Tokyo, Japan), according to the method of Takishima and colleagues.¹¹ Briefly, respiratory resistance was measured by using the forced oscillation method (3 Hz) during inhalation of methacholine continuously with stepwise incremental concentrations until respiratory resistance reached twice the baseline value. Methacholine chloride was diluted in physiologic saline solution and administered in 2-fold increasing concentrations from 49 to 25,000 µg/mL. Each concentration of methacholine solu-

tion was inhaled for a period of 1 minute. All subjects were examined during quiet breathing in a sitting position. Bronchial hyperresponsiveness was evaluated as the minimum cumulative dose (Dmin) of methacholine for which the reciprocal of respiratory resistance decreased linearly. Dmin was calculated so that 1 unit of Dmin equaled 1 minute of inhalation of aerosol solution at 1.0 mg/mL.

Bronchoscopy and tissue preparation

A bronchoscope (BF type 1T-30; Olympus Co, Tokyo, Japan) was inserted through the mouth, and biopsy forceps (FB-15C, Olympus) were used to collect 2 biopsy specimens from the subcarina of the right upper lobe. For in situ hybridization, the specimen was fixed immediately in freshly prepared 4% paraformaldehyde for 2 hours, washed twice (1 hour) with 15% sucrose in PBS, embedded in OCT, snap-frozen, and stored at -70°C to be used later. For immunocytochemistry, another specimen was immersed in 15% PBS and cooled on ice. After the tissue was blocked in OCT medium and frozen, cryostat sections (4 µm) were cut, air-dried for 1 hour, fixed by immersion in a mixture of acetone/methanol (60:40) for 7 minutes, stained with hematoxylin and eosin for conventional histologic examination (Fig 1, A), and stored at -20°C until further use.

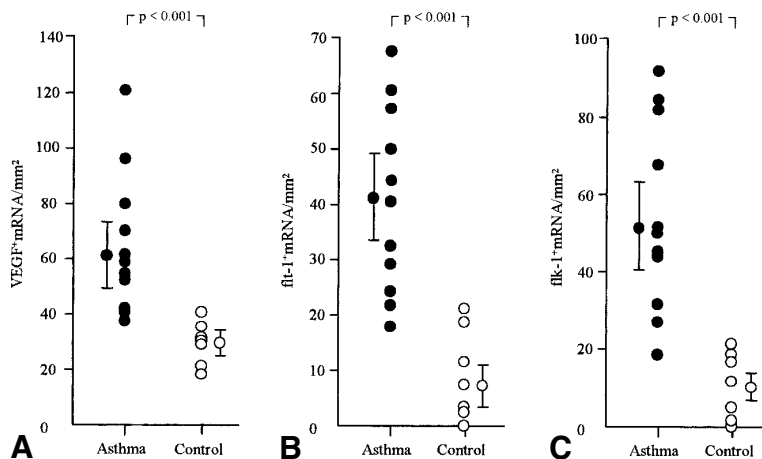


FIG 2. Number of VEGF (A), flt-1 (B), and flk-1 (C) mRNA⁺ cells per square millimeter of submucosa measured by means of in situ hybridization in bronchial biopsy specimens from asthmatic (n = 11) and control subjects (n = 8). Group values are presented as medians with interquartile ranges.

Immunocytochemistry

Immunocytochemistry was performed by using the alkaline phosphatase-antialkaline phosphatase method, as previously described.¹² The collagen-type IV mAb (dilution 1:50; Monosan, Uden, The Netherlands) was used to quantify the blood vessels within the bronchial mucosa. For negative controls, the primary antibody was replaced with nonspecific mouse Ig.

Probe preparation

Riboprobes were prepared from DNA encoding VEGF, flt-1, and flk-1 (a gift from Dr M. Shibuya, The University of Tokyo, Tokyo, Japan), as described previously.¹³ cDNAs were inserted into different pGEM vectors and linearized with restriction enzymes before transcription. Transcription was performed in the presence of sulfur 35-labeled uridine triphosphate and the appropriate T7 or SP6 RNA polymerase.

In situ hybridization

In situ hybridization on 4-μm cryostat sections was performed as previously described.^{14,15} Briefly, slides were first permeabilized by immersion in 0.3% Triton X-100 and proteinase K solution (1 μg/mL). Prehybridization was carried out in 50% formamide in 2× standard saline citrate (SSC) solution for 15 minutes at 37°C. Hybridization was performed by using ³⁵S-uridine triphosphate-labeled riboprobes (antisense) overnight at 42°C. Posthybridization washes were carried out by using SSC solutions (4× SSC to 0.1× SSC) followed by RNase treatment to remove unhybridized single-stranded RNA. The preparations were dehydrated, immersed in emulsion fluid, and subjected to autoradiography for 14 days. The autoradiograms were developed, and sections were counterstained with hematoxylin. As negative controls, sections were hybridized with the sense probes for VEGF, flt-1, and flk-1 or were pretreated with RNase before hybridization with the antisense probes.

Combined in situ hybridization and immunocytochemistry

The phenotype of cells expressing VEGF⁺, flt-1⁺, and flk-1⁺ mRNA was determined by using simultaneous in situ hybridization and immunocytochemistry, as previous described.^{12,16} Briefly, the sections were immunostained with mAbs directed against major basic protein (anti-major basic protein; Harlan Sera-lab Lt, Sussex,

United Kingdom), mast cell tryptase (anti-AA1; Dako Ltd, High-Wycomb, United Kingdom), T cells (anti-CD3; Becton Dickinson, Oxford, United Kingdom), macrophages (anti-CD68, Dako), and CD34⁺ cells (anti-CD34; Biomedica Corp, Foster, Calif). After visualization of the immunopositive cells with the alkaline phosphatase-antialkaline phosphatase technique, the sections underwent in situ hybridization for VEGF, flt-1, and flk-1 mRNA.

Quantitation

Slides were coded, and positive cells were counted blindly by using 200× magnification. A computerized colorimetric image analyzer (Win ROOF; Mitani Corp, Fukui, Japan) was used to quantify collagen-type IV immunoreactivity in tissue. The entire submucosa beneath the epithelial basement membrane was outlined on the color monitor by means of an electronic mouse cursor. All structures internal to the vessel endothelial basement membrane were evaluated as vascular area. The percentage of vascularity was determined by dividing the vascular area by the submucosal area, a more exact measure of angiogenic change than quantifying the number of vessels. The numbers of positively stained cells were counted, and the individual results were expressed as the total number of positive cells per square millimeter of mucosa, excluding smooth muscle, glands, and vessels.

Statistical analysis

Between-group comparisons were performed by using the Mann-Whitney *U* test with the Bonferroni correction. The same observer performed at least 3 replicate measurements, and intraobserver reproducibility was assessed by using the coefficient of variation for repeat measurements. The intraobserver coefficient of variation ranged from 4% to 15% for the percentage of vascularity and was less than 5% for the number of positively stained cells. Correlation coefficients were obtained by using Pearson linear regression analysis. A *P* value of less than .05 was considered significant.

RESULTS

Vascularity of airways

There was an increase in the percentage of vascularity in asthmatic patients compared with that in normal control subjects (median of 9.9% [range, 6.6%-24.3%] vs 2.7% [1.2%-4.5%], *P* < .01).

VEGF mRNA expression in the airways

VEGF mRNA was detected in bronchial biopsy specimens from asthmatic patients and control subjects (Fig 1, B and F). Bronchial biopsy specimens from asthmatic patients had significantly elevated numbers of cells expressing mRNA for VEGF compared with biopsy specimens from normal subjects (median of 61 cells/mm² [range, 39-122 cells/mm²] vs 29 cells/mm² [range, 19-41 cells/mm²], $P < .001$; Fig 2, A). There was a significant correlation between the degree of vascularity and the number of VEGF mRNA⁺ cells in bronchial biopsy specimens ($r = 0.97$, $P < .001$; Fig 3, A). The number of cells expressing VEGF mRNA correlated inversely with airway caliber (FEV₁; $r = -0.83$, $P < .01$) and with Dmin in subjects with asthma ($r = -0.97$, $P < .001$).

flt-1 and flk-1 mRNA expression in the airways

The mRNAs of the VEGF receptors were expressed in bronchial biopsy specimens from asthmatic patients and control subjects (Fig 1, C and D). A significant increase in the number of cells expressing mRNA for both flt-1 (median of 41 cells/mm² [range, 17-67 cells/mm²] vs 7 cells/mm² [range, 0-21 cells/mm²], $P < .001$) and flk-1 (52 cells/mm² [range, 19-91 cells/mm²] vs 12 cells/mm² [range, 0-21 cells/mm²], $P < .001$) was detected in the biopsy specimens from asthmatic subjects compared with normal subjects (Fig 2, B and C). The number of cells expressing flt-1 mRNA ($r = 0.61$, $P < .05$) and flk-1 mRNA ($r = 0.67$, $P < .05$) correlated with the percentage of vascularity (Fig 3, B and C). The number of cells expressing VEGF mRNA also correlated significantly with the number of flt-1 mRNA⁺ cells ($r = 0.89$, $P < .01$) and flk-1 mRNA⁺ cells ($r = 0.88$, $P < .01$).

Cell sources of VEGF, flt-1, and flk-1

Colocalization studies were performed in bronchial biopsy specimens from asthmatic patients ($n = 8$) by using combined in situ hybridization and immunocytochemistry (Fig 1, E and G). In the mucosa, CD68⁺ (mean $\pm$ SEM, 28.1% $\pm$ 6.7%), major basic protein–positive (20.7% $\pm$ 5.6%), and CD34⁺ (20.2% $\pm$ 6.9%) cells were the major cell types expressing VEGF. Furthermore, the majority of flt-1 and flk-1 mRNA⁺ cells were associated with CD34⁺ (47.4% $\pm$ 2.2% expressing flt-1 mRNA⁺ and 45.0% $\pm$ 8.5% expressing flk-1 mRNA⁺), CD68⁺ (26.4% $\pm$ 3.0% expressing flt-1 mRNA⁺ and 28.7% $\pm$ 5.0% expressing flk-1 mRNA⁺), and CD3⁺ (15.0% $\pm$ 3.5% expressing flt-1 mRNA⁺ and 17.0% $\pm$ 4.1% expressing flk-1 mRNA⁺) cells.

DISCUSSION

The results of this study demonstrate that the expression of VEGF and both of its receptors is upregulated within the airways of asthmatic patients compared with those of control subjects and that the expression of VEGF correlates with the degree of submucosal vascularity. In addition, VEGF expression was inversely correlated with

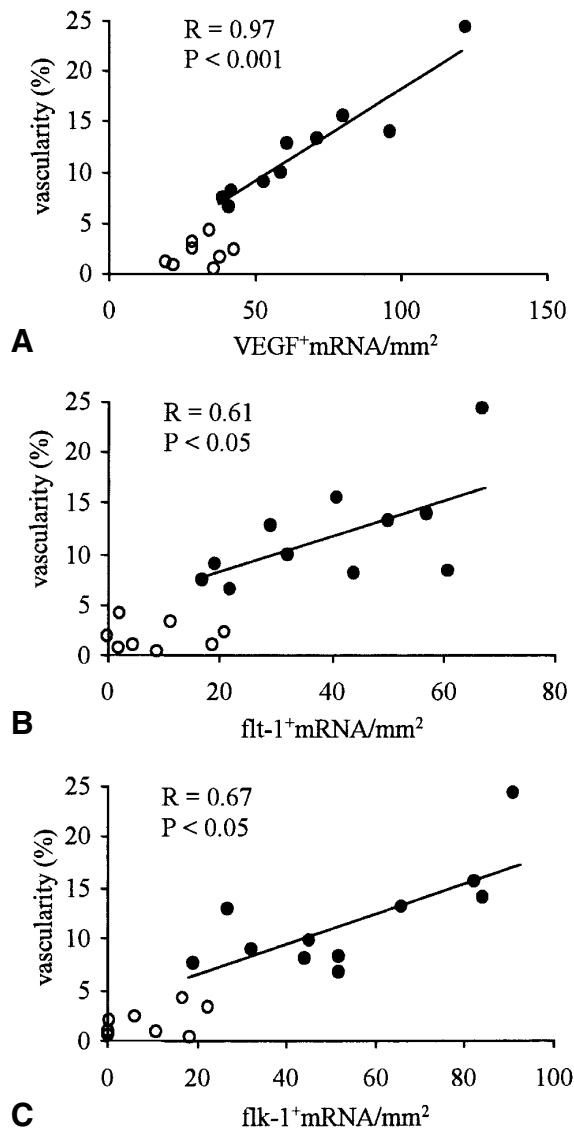


FIG 3. Correlation between the percentage of vascularity and VEGF mRNA⁺ (A), flt-1 mRNA⁺ (B), and flk-1 mRNA⁺ (C) cells. Correlations are significant only for asthmatic patients. Filled circles, Asthmatic patients; open circles, control subjects.

FEV₁ measurements and airway hyperresponsiveness. This is the first in vivo study characterizing the mRNA expression of VEGF and its receptors and the association of VEGF with airway vascularity in bronchial asthma.

The contribution of vascularity to airway remodeling has not been fully elucidated. The findings of increased vascularity in the asthmatic mucosa in this study and others^{4,5} suggest that angiogenesis is a component of the chronic inflammatory response, even in subjects with mild-to-moderate asthma.

We report that the increase in the number of cells expressing VEGF mRNA correlates significantly with reduced airway caliber and airway responsiveness. This finding is consistent with a previous report demonstrating a significant inverse correlation between airway responsive-

ness and vessel number in the lamina propria of asthmatic airways.¹⁷ Our findings suggest that the release of VEGF from airways may contribute to both the development of airway vascularity and the decline in lung function.

The most important role of VEGF in inflammation may be in mediating the angiogenic response.^{18,19} Recently, it has been reported that VEGF is also overexpressed in other chronic inflammatory diseases, such as rheumatoid arthritis²⁰ and nasal polyp tissues.²¹ Demoly et al²² compared the level of VEGF in bronchoalveolar lavage fluid of chronic stable asthmatic subjects, patients with chronic bronchitis, and control subjects. They found that there was no significant difference in any of the subject groups. The difference between our results and those of Demoly et al may reflect differences in the study method; that is, the VEGF detected in bronchoalveolar lavage fluid may not represent bronchial mucosa expression of VEGF.

Previous reports indicated that VEGF is expressed by many cell types, including epithelial cells,²³ mononuclear cells, keratinocytes,²⁴ macrophages, eosinophils,²⁵ and smooth muscle cells.^{23,26} In this study, with the use of simultaneous *in situ* hybridization and immunocytochemistry, we demonstrated that macrophages, eosinophils, and CD34⁺ cells within the bronchial mucosa of asthmatic individuals were the major sources of cells expressing VEGF. Both VEGF mRNA and protein are overexpressed by the synovial macrophages lining joints and inflamed rheumatoid synovial tissues.²⁰ More recently, it has been reported that eosinophils may be an important source of VEGF.²⁵ Furthermore, phenotyping the cells expressing flt-1 and flk-1 revealed that CD34⁺ cells, macrophages, and T cells were the major cellular sources of VEGF receptors in asthmatic individuals. A marker, the CD34 antigen, has been used to identify hematopoietic precursor cells. Recently, both flt-1 and flk-1 expression has been described in hematopoietic precursor cells.^{27,28} We did not include specimens from atopic nonasthmatic subjects, and thus it is not clear whether VEGF and its receptors are upregulated in accordance with atopy, or asthma, or both.

In conclusion, we have demonstrated that VEGF, flt-1, and flk-1 mRNAs are overexpressed in bronchial asthma and are associated with the degree of airway vascularity. In this study only mRNA expression was examined. Further studies are necessary to confirm the presence of VEGF protein and its receptor proteins in asthmatic airways.

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