

Vascular endothelial growth factor up-regulation and bronchial wall remodelling in asthma

A. Chetta*, A. Zanini†, A. Foresi‡, R. D'Ippolito*, A. Tipa*, A. Castagnaro*, S. Baraldo§, M. Neri†, M. Saetta§ and D. Olivieri*

*Department of Clinical Sciences, Section of Respiratory Diseases, University of Parma, Parma, Italy, †Salvatore Maugeri Foundation, Division of Pneumology, IRCCS Rehabilitation Institute of Tradate, Gussago, Italy, ‡Respiratory Pathophysiology Laboratory, Sesto S. Giovanni Hospital, Sesto San Giovanni, Italy and §Department of Clinical and Experimental Medicine, Section of Respiratory Diseases, University of Padua, Padua, Italy

Summary

Background There is increasing *in vitro* evidence to support a role for vascular endothelial growth factor (VEGF), a major regulator of angiogenesis, as a mediator of fibrosis associated with neovascularization.

Objective We tested the hypothesis that VEGF is involved both in increased airway mucosal vascularity and in the subepithelial fibrosis of asthmatic patients.

Methods Bronchial biopsies were performed in 24 asthmatic patients and eight healthy controls. Immunostaining, using computerized image analysis, was performed using monoclonal antibodies against VEGF⁺ cells, type IV collagen, to outline the basement membrane thickness, and tryptase and EG2, to identify mast cells and eosinophils, respectively.

Results The counts of VEGF⁺ cells ($P < 0.05$), mast cells and EG2⁺ cells (both $P < 0.01$) were higher in asthmatics than in controls. The number of vessels, the vascular area in the lamina propria, and the basement membrane thickness were significantly higher in asthmatics than in healthy volunteers ($P < 0.01$). Moreover, in asthmatic patients, the number of VEGF⁺ cells was significantly related to the number of vessels ($P < 0.01$), to mast cells ($P < 0.01$) and to basement membrane thickness ($P < 0.01$). A colocalization study also revealed that mast cells were a relevant cellular source of VEGF. High doses of inhaled fluticasone propionate significantly reduced VEGF⁺ cells ($P < 0.05$), vessel number ($P < 0.05$), vascular area ($P < 0.05$) and basement membrane thickness ($P < 0.05$) in a subgroup of asthmatic patients.

Conclusions This study shows that VEGF, in addition to being involved in the vascular component of airway remodelling, may play a role in the thickening of the basement membrane in asthma.

Keywords airway remodelling, asthma, bronchial biopsy, vascular endothelial growth factor

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Introduction

Asthma is a chronic inflammatory disease characterized by airway wall remodelling. The thickening of the reticular basement membrane, along with an abnormal proliferation of mucosal blood vessels and airway muscle cells, is the main contributor to this process [1].

There is increasing evidence to support a key role for vascular endothelial growth factor (VEGF), a major regulator of angiogenesis and enhancer of vascular permeability [2], as a mediator of structural changes in the airway wall of asthmatics.

Asthmatic subjects exhibit a greater expression of VEGF, as compared with control subjects, and colocalization studies show that macrophages, eosinophils and CD34⁺ cells are the major sources of VEGF [3]. In asthmatic subjects, VEGF

expression is strictly correlated with an increased vascularity [3]. Moreover, in a murine model, toluene diisocyanate-induced asthma was characterized by airway hyper-responsiveness, inflammation and increased VEGF levels [4]. The administration of VEGF receptor inhibitors reduced all these pathophysiological aspects [4].

Furthermore, *in vitro* studies suggest that VEGF expression could induce fibrosis associated with neovascularization. In particular, VEGF treatment enhanced the production of matrix metalloproteinases by human vascular smooth muscle cells [5] and the expression of connective tissue growth factor in bovine retinal endothelial cells and pericytes [6]. Recently, it has been also shown that VEGF could induce fibronectin secretion by human airway muscle cells [7].

Therefore, we hypothesized that in asthmatic airways, an up-regulated VEGF expression may be associated not only with an increased vascularity, but also with subepithelial fibrosis. To test our hypothesis we compared VEGF expression in bronchial biopsy specimens from patients with mild-

Correspondence: Prof. Alfredo Chetta, Clinica Pneumologica Azienda Ospedaliero-Università di Parma, Viale G. Rasori, 10 - 43100 - Parma, Italy.
E-mail: chetta@unipr.it

to-moderate asthma against normal control subjects and examined the relationship between VEGF and airway wall inflammation and remodelling. Data from this study have been previously published in abstract form [8].

Methods

Subjects

Twenty-four non-smoking patients with mild-to-moderate asthma were recruited from the outpatient clinic of the Department of Clinical Sciences of the Parma University. All had a well-documented history of asthma [9], and baseline forced expiratory volume in 1 s (FEV₁) had to be 70% or more of predicted. All patients were free from asthma attacks in the previous 2 months and controlled their symptoms with inhaled salbutamol only. They did not receive corticosteroids for 6 months prior to the study and were free from respiratory infections in the 4 weeks preceding the study. The degree of asthma severity was assessed according to an asthma severity score based on symptoms, bronchodilator use and daily peak expiratory flow variability, measured during the 3 weeks prior to the methacholine challenge test day. Possible scores ranged from 0 to 12 [10]. Atopy was assessed by skin prick tests with a battery of 10 common inhalant allergens. Eight healthy non-smoking volunteers were enrolled, as a control group. The study was approved by the University of Parma ethical committee, and subjects gave written informed consent.

Spirometry and methacholine challenge

Baseline FEV₁ was measured in triplicate by a flow-sensing spirometer connected to a computer for data analysis (Vmax 22, Sensor Medics, Yorba Linda, CA, USA). Methacholine inhalation challenge was done according to the European Respiratory Society guidelines [11]. Doubling increasing concentrations of methacholine from 0.03 to 32 mg/mL were delivered by a dosimeter (output: 9 µL/puff; Mefar, Brescia, Italy) and inhaled. Inhalations were interrupted when FEV₁ decreased by 20% from its post-saline value. The concentration provoking a 20% decrease in FEV₁ (PD methacholine [PD₂₀ M], in µmol) was determined by linear interpolation of the last two experimental points.

Fibre-optic bronchoscopy

Fibre-optic bronchoscopy was performed according to a previously described protocol [10]. Premedication consisted of atropine (0.5 mg) and diazepam (10 mg), both given by intramuscular injection. Local anaesthesia was obtained with a tetracaine tablet (20 mg) given 15 min before bronchoscopy. An additional aliquot of 2% lignocaine was aerosolized into the upper airways and applied topically to the pyriform sinuses and vocal cords to prevent coughing and as a local anaesthetic. Nebulized salbutamol (1.25 mg) and ipratropium bromide (0.25 mg) were inhaled 5 min prior to bronchoscopy (Model 1T10; Olympus, Tokyo, Japan). Three to five mucosal biopsy specimens were taken at the subcarinae in the middle and lower lobes of the right lung. Administration of nebulized salbutamol and ipratropium bromide was repeated after bronchoscopy when necessary. Patients were closely mon-

itored after bronchoscopy in the outpatient clinic, and were discharged when the effects of sedation disappeared and lung function returned to the baseline values.

Biopsy processing

Mucosal biopsies were immediately transferred into ice-cold acetone containing the protease inhibitor iodoacetamide (20 mM) and phenylmethylsulphonylfluoride (2 mM) for fixation; they were then stored at -20 °C for 24 h and processed into the water-soluble resin, glycolmethacrylate (Polysciences, Northampton, UK), for embedding [12].

Sections of 2 µm were cut and stained using monoclonal antibodies against type IV collagen to outline the endothelial basement membrane (Novocastra Lab., Newcastle, UK), tryptase (DAKO, Glostrup, Denmark) and EG2 (Pharmacia and Upjohn Diagnostic AB, Uppsala, Sweden) to identify mast cells and eosinophils, respectively, and VEGF (NeoMarkers, Fremont, CA, USA) to recognize the 121, 165 and 189 isoforms of VEGF. Light microscopy was performed with a Leica microscope (Leica DMLB, Werzlar, Germany) at × 1000 magnification.

The number of vessels, mast cells, eosinophils and VEGF⁺ cells were counted in all non-overlapping high-power fields in the lamina propria. The latter is defined as a zone 100 µm below the epithelial basement membrane, and expressed as number of vessels and cells per square millimetre of lamina propria. In each section, all available non-overlapping high-power fields with intact tissue covered by intact basement membrane were examined. Vascular area was expressed as percentage of the area of the assessed lamina propria. The mean size of vessels was estimated by dividing the total vascular area by the total number of vessels. Basement membrane thickness was measured as described previously [13, 14]. Final values represent the mean of at least two sections from two different biopsies in each subject. A single observer who had no knowledge of patient characteristics evaluated tissue sections using an image analysis system (Leica Q500C; Leica Cambridge Ltd, Cambridge, UK). An average of eight power fields was examined for each subject included in the study. The mean coefficient of variation for repeated measurements was 10% for number of vessels and vascular area, 12% for VEGF⁺ cells, 6% for mast cells, 7% for eosinophils and 10% for basement membrane thickness.

In a subgroup of six asthmatic patients, the number of VEGF⁺ cells, the number and the size of vessels, the vascular area and the basement membrane thickness were examined after a 6-week course of high dose inhaled fluticasone propionate.

Statistical analysis

Values are presented as mean ± standard deviation (SD), unless otherwise specified. The unpaired *t*-test, the Mann-Whitney rank sum test and the χ^2 test were used for comparison between patients with asthma and control subjects, when appropriate. Relationships were estimated by Spearman's rank correlation coefficient (*r*_s). A *P*-value ≤ 0.05 was taken as significant.

Table 1. Characteristics of asthmatic and normal control subjects

	Asthmatic subjects (<i>n</i> = 24)	Control subjects (<i>n</i> = 8)
Age (years)	29 ± 10	25 ± 3
Sex (F:M)	6:18	4:4
Duration of disease (years)	9 ± 8	—
FEV ₁ (% predicted)	106 ± 18	108 ± 8
PD ₂₀ methacholine (μmol)	1.15 ± 0.6	>40
Asthma severity score (0–12)	5.5 (3–10)	—

FEV₁, forced expiratory volume in 1 s; F, female; M, Male.

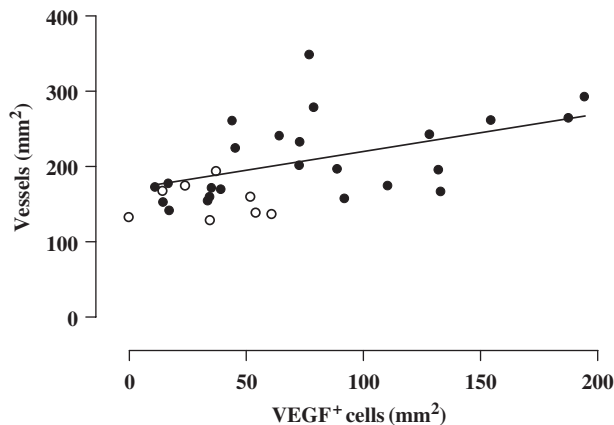


Fig. 1. Correlation between the number of vessels and the number of cells expressing VEGF. Closed circles = patients with asthma ($r_s = 0.56$, $P < 0.01$); open circles = control subjects ($r_s = -0.02$, $P = \text{NS}$).

Results

Characteristics of asthmatic patients and normal control subjects are reported in Table 1. According to the asthma severity score, patients with asthma had persistent mild-to-moderate asthma (score range 3–10, median value 5.5).

Fibre-optic bronchoscopy with endobronchial biopsy specimens was performed successfully and was well tolerated in all subjects. Patients with asthma significantly differed from healthy volunteers in VEGF⁺ cells (78 ± 54 vs. 35 ± 21 cells/mm², $P < 0.05$), in number of vessels (209 ± 54 vs. 153 ± 23 vessels/mm², $P < 0.01$) and in vascular area ($5.2 \pm 1.6\%$ vs. $3.0 \pm 0.7\%$, $P < 0.01$). There was no significant difference in mean vessel size (225 ± 53 vs. 203 ± 31 μm²) in patients with asthma, as compared with control subjects. In addition, when compared with healthy volunteers, patients with asthma had significantly higher values for basement membrane thickness (9.4 ± 1.2 vs. 8.0 ± 0.2 μm, $P < 0.01$), number of mast cells (65 ± 27 vs. 35 ± 9 cells/mm², $P < 0.01$) and number of activated eosinophils (101 ± 80 vs. 31 ± 22 cells/mm², $P < 0.01$).

In patients with asthma, the number of VEGF⁺ cells significantly correlated with the number of vessels ($r_s = 0.56$, $P < 0.01$) (Fig. 1), the number of mast cells ($r_s = 0.56$, $P < 0.01$) (Fig. 2) and with the basement membrane thickness ($r_s = 0.60$, $P < 0.01$) (Fig. 3). There was also a weak correlation between the number of VEGF⁺ cells and the duration of disease ($r_s = 0.41$, $P < 0.05$). Finally, the number of mast cells was significantly related to the number of vessels ($r_s = 0.87$, $P < 0.01$) and to the vascular area ($r_s = 0.47$, $P < 0.05$). Asthma symptom score, baseline FEV₁ and PD₂₀

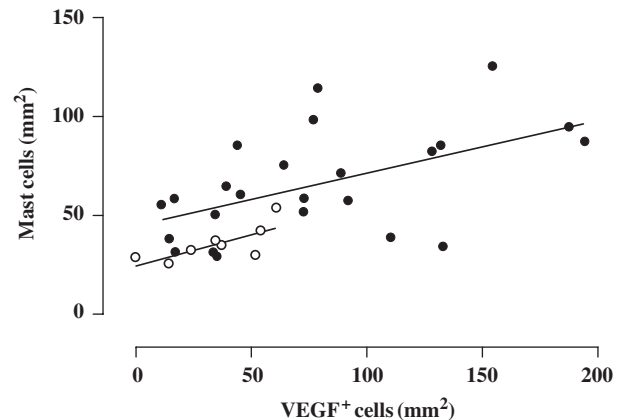


Fig. 2. Correlation between the number of mast cells and the number of cells expressing VEGF. Closed circles, patients with asthma ($r_s = 0.56$, $P < 0.01$); open circles, control subjects ($r_s = 0.81$, $P < 0.05$).

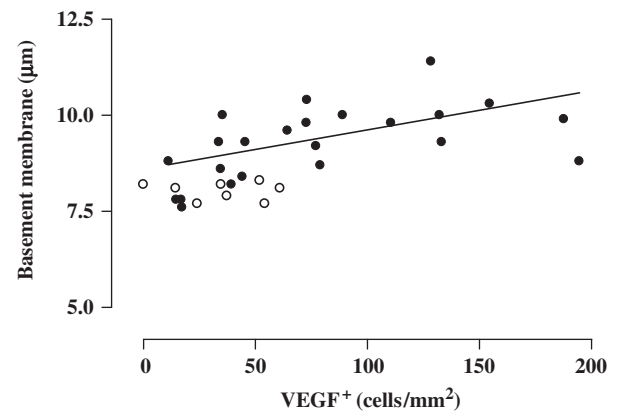


Fig. 3. Correlation between the basement membrane thickness and the number of cells expressing VEGF. Closed circles, patients with asthma ($r_s = 0.60$, $P < 0.01$); open circles, control subjects ($r_s = -0.16$, $P = \text{NS}$).

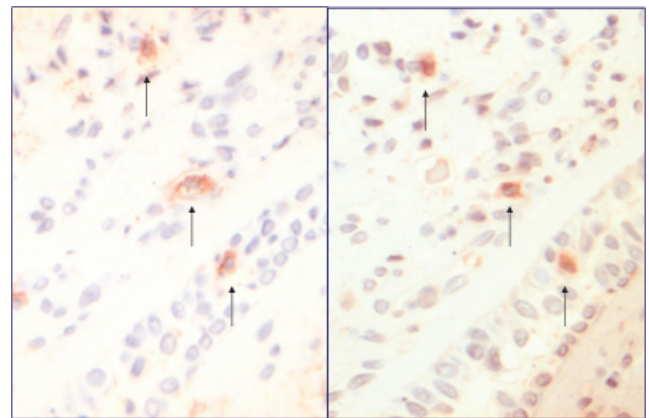


Fig. 4. Microphotograph showing sequential 2 μm sections of bronchial mucosa from an asthmatic patient stained with antibodies to VEGF (right panel) and to mast cells (left panel). Arrows show cells stained with both antibodies, indicating colocalization.

M values were not significantly related to the number of VEGF⁺ cells.

Examination of serial biopsy sections was performed in a subgroup of 12 asthmatic patients and comparison of

Table 2. Mean $\pm$ SD values of VEGF, number and size of vessels, vascular area and basement membrane thickness in six asthmatic patients before and after a 6-week course of inhaled fluticasone propionate high doses ($860 \pm 320 \mu\text{g}$ daily)

	Pre	Post	P
VEGF ⁺ (cells/mm ²)	86.4 $\pm$ 42	68.1 $\pm$ 28	0.046
Vessels/mm ²	220 $\pm$ 37	165 $\pm$ 32	0.028
Vessel size (μm^2)	241 $\pm$ 52	208 $\pm$ 15	0.116
Vascular area (%)	5.4 $\pm$ 1.3	3.5 $\pm$ 0.6	0.028
Basement membrane thickness (μm)	9.7 $\pm$ 1.2	8.2 $\pm$ 0.8	0.046

VEGF, vascular endothelial growth factor.

adjacent sections showed that in the lamina propria a median of 24.5% (range 13.1–44.8%) of mast cells colocalized with VEGF⁺ (Fig. 4).

In normal control subjects, the number of VEGF⁺ cells significantly correlated with the number of mast cells ($r_s = 0.81$, $P < 0.05$) (Fig. 2), but not with the basement membrane thickness (Fig. 3).

In the subgroup of six asthmatic patients (age range: 18–42 years, asthma severity score range: 4–10) who received a 6-week course of inhaled fluticasone propionate with a daily average dose of $860 \pm 320 \mu\text{g}$, the number of VEGF⁺ cells, the number of vessels, the vascular area and basement membrane thickness after treatment were significantly reduced, as compared with baseline values (Table 2).

Discussion

This study confirmed that the expression of VEGF is up-regulated in the bronchial mucosa of mild-to-moderate asthmatic patients compared with that of control subjects, and demonstrated that it is related to the number of vessels and mast cells as well as to the basement membrane thickness. Taken together, these results suggest that VEGF may play a key role not only in bronchial mucosa vascular changes, but also in airway wall inflammation and remodelling.

Several growth factors with proven or potential roles in vascular development and remodelling have been identified and have been associated with various disease conditions [15]. In the present study, we focused on the expression of VEGF, which is a highly specific mitogen for endothelial cells *in vitro* and has angiogenic properties *in vivo* [16]. Moreover, VEGF has been reported to have a narrow target cell range and is specific for endothelial cells, because VEGF binds to the high-affinity cell surface receptors, predominantly expressed by endothelial cells [17, 18]. The expression of VEGF in airways of asthmatic patients has been previously examined. Demoly et al. [19] compared the VEGF level in bronchoalveolar lavage (BAL) fluid of patients with stable asthma, patients with chronic bronchitis and control subjects. They found that there was no significant difference in VEGF levels among these three study groups. However, the VEGF detected in BAL fluid may not represent bronchial mucosa expression of VEGF, as BAL mainly assesses distal airways and alveoli. Indeed, other studies [20, 21] showed that the concentration of VEGF in induced sputum was significantly higher in asthmatic patients than in normal controls. In bronchial

biopsy specimens from asthmatic patients, we found that the VEGF expression was increased as compared with that of healthy control subjects. Our data are consistent with previous results by Hoshino et al. [3, 22]. Interestingly, in spite of differences in inclusion techniques between our study and Hoshino et al. [22] studies, the magnitude of VEGF expression, both in asthmatic and in control subject airways, was similar. However, unlike the Hoshino study, we did not find any relationship between airway calibre or degree of bronchial responsiveness to methacholine and VEGF expression. This discrepancy may be explained by the difference in patient selection, as all our patients, at variance with those of Hoshino's study, had mild asthma with normal baseline lung function.

In this study, we found that, in asthmatic patients, the number of VEGF⁺ cells was significantly correlated to the number of vessels, but not to the vascular area nor to the vessel size. Our data suggest that VEGF has angiogenic properties in asthma, rather than enlarging the pre-existing mucosal blood vessels. In mild-to-moderate asthmatic patients, previous studies found a significant correlation between VEGF expression and mucosal airway vascularity [3, 22]. Indeed, VEGF is a potent multi-functional cytokine with several important effects and it can act on angiogenic sprouting as well as on vascular leak and permeability [2]. Several inflammatory cells are considered capable to release VEGF [3]. The colocalization analysis performed in this study demonstrated that in asthmatics, mast cells were a relevant cellular source of VEGF. Moreover, we found a significant relationship between the number of VEGF⁺ cells and the number of mast cells, both in asthmatic and in healthy subjects. Our findings further support the view that mast cells can release VEGF and contribute to the neovascularization process in asthma. Indeed, it has been demonstrated *in vitro* that mast cells can produce and secrete VEGF [23], which in turn has been shown to stimulate mast cell migration to sites of angiogenesis [24]. In addition, histamine, the major preformed mast cell mediator, stimulates new vessel growth by acting through both H₁ and H₂ receptors [25], and heparin, the main glycosaminoglycan constituent of mast cells granules, demonstrates pro-angiogenic activity [26]. The positive correlation between mast cells and the number of vessels observed in asthmatic patients [27] further supports these observations.

As expected, we found that in asthmatic patients the basement membrane was thicker than that in healthy controls. The subepithelial collagen thickening may be considered part of the inflammatory response to the offending agents, as well as a marker of long-lasting structural changes in the airway wall, likely related to the chronic nature of the disease [1]. Hoshino et al. [28] evaluated the relationships between airway wall remodelling and the expression of cellular growth factors. They found that, although the subepithelial layer was thicker in asthmatic patients when compared with that of normal subjects, the expression of each growth factor was similar. By contrast, in the present study, we found an increased VEGF expression in asthmatic patients, which was correlated to subepithelial basement membrane thickness. Previous *in vitro* studies showed that VEGF expression can be associated with fibrosis and the remodelling processes. Suzuma et al. [6] found that VEGF can increase the expression of connective tissue growth factor, a growth factor

that acts on fibroblast proliferation and matrix production. Furthermore, Kazi et al. [7] showed that VEGF could stimulate fibronectin secretion from human airway muscle cells. Interestingly, there are recent *in vivo* observations which confirm these data. Indeed, Lee et al. [29] using VEGF transgenic mice clearly demonstrated that VEGF is a potent inducer both of angiogenesis and subepithelial fibrosis. To the best of our knowledge, our study provides the first evidence that VEGF expression is associated with subepithelial fibrosis in humans as well. Taken together, these findings suggest that VEGF may contribute to airway wall remodelling in patients with asthma not only by acting on angiogenesis, but also by affecting the extracellular matrix composition and by stimulating fibrotic changes.

The mechanism by which VEGF could induce subepithelial fibrosis in asthmatic patients is not yet defined and unfortunately our study was not specifically designed to investigate this mechanism. However, it has been recently shown that VEGF has a critical role in enhancing chronic TH2 inflammation and TGF- β_1 production [29], which in turn may result in airway subepithelial fibrosis. Furthermore, TGF- β_1 administration in mice has been shown to promote peribronchial collagen deposition [30]. It is therefore conceivable that in humans as well, VEGF may enhance chronic airway inflammation and promote airway subepithelial fibrosis through a TGF- β_1 -mediated pathway. Even if from our study we cannot draw any conclusion on mechanisms of basement membrane thickening in humans, it is interesting to note that, in a subgroup of patients, inhaled fluticasone significantly reduced basement membrane thickness, VEGF expression and mucosal microvasculature. The observation that VEGF up-regulation was reversed along with airway structural changes could indicate that VEGF expression and airway remodelling could be linked by a mechanism sensitive to corticosteroids.

In summary, we found that in asthmatic patients, up-regulated VEGF expression may be associated with increased vascularity, as well as with subepithelial fibrosis. Our results further support the view that in bronchial asthma, angiogenesis and fibrosis can be considered as two key components in wound healing and tissue regeneration of bronchial airways.

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