

ORIGINAL PAPER

Airway angiogenesis in patients with rhinitis and controlled asthma

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Clinical and
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Summary

Background Airway angiogenesis may be an important part of structural remodelling in the pathogenesis of asthma. The development of asthma is frequently preceded by rhinitis.

Objective We sought to determine whether the levels of angiogenesis-related factors are elevated in airways of patients with rhinitis or controlled asthma.

Methods We analysed the induced sputum of 18 rhinitis patients, 16 asthmatic patients, and 15 healthy controls. The concentrations of angiogenin, vascular endothelial growth factor (VEGF), IL-8, fibroblast growth factor (bFGF), and TNF- α were measured by cytometric bead arrays.

Results We found significantly increased angiogenin and VEGF concentrations in the induced sputum supernatant of both rhinitis and asthma patients compared with that of the healthy control group ($P \leq 0.0005$). With the exception of TNF- α , there was no difference in the other angiogenic factors; TNF- α levels were higher in the rhinitis group than in the control group ($P = 0.02$).

Conclusion These *in vivo* results suggest increased airway angiogenesis in patients with rhinitis without asthma as well as in corticosteroid-treated and well-controlled asthma patients.

Keywords angiogenesis, asthma, rhinitis, sputum

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Introduction

The presence of an inflammatory response and airway remodelling are cornerstones in the pathogenesis of asthma [1, 2]. Airway remodelling is thought to lead to irreversible airway obstruction, a feature that is more prominent in patients with severe disease, and may even persist following anti-inflammatory treatment [1]. The functional sequelae of airway remodelling in asthmatics are believed to include the development of non-specific airway responsiveness, accelerated decline in lung function, and loss of the post-bronchodilator response to β_2 -adrenoreceptor agonists [3]. Airway wall remodelling in asthma involves a number of changes, including changes at the level of the blood vessels. A number of investigators have highlighted prominent increases in vessel number, vessel size, vascular surface area, and microvascular leakage [1, 4–9]. It appears that an imbalance in favour of angiogenic factors leads to the abnormal growth of new blood vessels in asthma patients [10]. Angiogenesis is a complex process, and vascular endothelial growth factor

(VEGF) is one of the most potent pro-angiogenic factors. It is expressed in highly vascularized organs and tissues like the lung, and it acts to stimulate endothelial cell migration and proliferation [11, 12]. Very recently, Simcock et al. [13] demonstrated that VEGF present in the asthmatic airway induces angiogenesis *in vitro*. Other growth factors involved in the process of angiogenesis include angiogenin, fibroblast growth factor (bFGF), IL-8, and TNF- α . Angiogenin was first isolated from conditioned medium from colonic carcinoma cell cultures [14, 15]. *In vivo*, angiogenin induces vascular endothelial cell proliferation, migration, and tubule formation [16]. IL-8, a chemokine that is produced by the bronchial epithelium and macrophages and is a potent neutrophil activator and chemoattractant, was found in elevated concentrations in the lungs of severely asthmatic patients. Increased levels of IL-8 and neutrophil number were also found in tracheal aspirates of patients intubated due to severe asthma exacerbation [17–21]. bFGF is a potent mitogen for many cell types, including airway smooth muscle cells, fibroblasts, and endothelial cells [22].

TNF- α is a cytokine that is produced by many cells (e.g. macrophages, T cells, mast cells, and epithelial cells), and its involvement has been suggested in asthmatic airway remodelling [23–26].

Few studies have investigated the essential role of angiogenesis in the pathogenesis of rhinitis. The findings from previous studies indicate that hypervascularity and VEGF in nasal mucosa might play important roles in the angiogenic process of allergic rhinitis (AR) [27–29].

Studies have also identified a temporal relationship between the onset of rhinitis and asthma, with rhinitis frequently preceding the development of asthma [30, 31]. In light of the known temporal relationships between the onset of rhinitis and asthma, there is a lack of data regarding the possible imbalance of major angiogenic factors in the airways of patients with rhinitis without concomitant asthma. We therefore sought to determine whether the levels of angiogenin, VEGF, IL-8, bFGF, and TNF- α are elevated in the induced sputum of patients with rhinitis without symptoms of asthma. The same angiogenic profile was examined in the induced sputum of asthmatic patients who were well controlled on their inhaled corticosteroid treatment.

Materials and methods

Study subjects

This study included 16 patients with stable asthma (Table 1). Asthma was diagnosed according to the Global Initiative for Asthma (GINA) [32]. According to the GINA guidelines, all patients had controlled asthma based on treatment with their inhaled corticosteroid therapy. Asthmatic patients were regularly observed and treated by a specialist for pulmonary diseases; according to the clinical symptoms, signs, and spirometry, their disease was well controlled and the patients were free of systemic corticosteroid therapy in the year before examination. We also demonstrated allergic sensitization in nine patients in the asthma group. There were 18 patients in the group of patients with rhinitis (Table 2). Rhinitis was diagnosed according to an ARIA workshop report [31, 33]. Among patients with rhinitis, 12 demonstrated allergic sensitization as assessed by the skin prick test (Allergopharma Ltd, Reibek, Germany) performed according to reported guidelines [33]. Among patients with rhinitis, 13 lacked any clinical symptoms or signs of asthma. In the remaining five patients, airway hyperresponsiveness was suspected and the PD 20 for metacholine was calculated. In all five cases, the PD 20 for metacholine was negative (below 4 mg cumulative dose). In the control group, we included 15 healthy subjects (nine female and six male) with a mean age of 30.8 years and normal pulmonary function tests [mean VC 4960 mL – 109%, mean forced expiratory volume in 1 s (FEV₁) 4088 mL – 105%]. Asthmatic patients

were older than patients with rhinitis and healthy subjects (one-way ANOVA), but there was no difference in age between patients with rhinitis and healthy subjects. There were no differences between the control group and the others in terms of sex, FEV₁, smoking, or atopy.

All subjects were free of symptoms and signs of acute upper respiratory tract infection in the month preceding the study. The patients were free of all other systemic diseases and malignancies. All subjects were volunteers and gave their written consent after being fully informed about the purpose and nature of the study. The study was approved by the national ethics committee for human studies.

Sputum induction and processing

Sputum was induced and processed according to previously described methods [34, 35]. Briefly, subjects inhaled 4.5% hypertonic saline, nebulized via an ultrasonic nebulizer (PARI MASTER Type 84.0100; PARI GmbH, Starnberg, Germany), for three 5-min periods. To minimize saliva contamination, we asked the subjects to mouthwash thoroughly and expectorate saliva in a separate container before producing sputum. At least 2 mL of sputum was collected into a sterile container. The collected sputum was pooled and immediately processed. The volume of the entire sputum sample was determined, and an equal volume of 0.1% dithiothreitol (Sputolysin R; Calbiochem, San Diego, CA, USA) was added. The samples were then mixed gently with a vortex mixer and incubated for 15 min at room temperature to ensure complete homogenization. After filtration and centrifugation, cell-free supernatants were frozen at –80 °C until subsequent analysis. The total number of non-squamous cells (TNNCs) per millilitre of the sputum sample was assessed using a haemocytometer. Cytospins were stained according to the May–Grünwald–Giemsa and Papnicolaou methods. Differential cell counts were performed by one observer, who counted 200 non-epithelial cells.

The quality of the induced sputum was assessed according to the recommendations of Pizzichini *et al.* [34]. Only samples with a score of 7 or higher were used for further analysis. There were no differences in the level of squamous cell contamination between the study groups.

Detection of angiogenic mediators

The detection of angiogenic mediators was performed as reported previously [36–38]. Angiogenin, VEGF (subtypes 165 and 121), IL-8, bFGF, and TNF- α concentrations were measured with Cytometric Bead Arrays (BD Biosciences, San Diego, CA, USA), which contain microparticles that are dyed to five different fluorescence intensities. Each particle is coupled with an antibody against one of five angiogenic cytokines: angiogenin, VEGF, IL-8, bFGF, and

Table 1. Clinical characteristics of patients with controlled asthma

Patient no.	Age	Sex	FEV ₁ % of predicted	FEV ₁ /VC	PC 20 (mg of methacholine/mL)	Smoking	Atopy	Inhaled corticosteroids (µg/day)	Long-acting β ₂ -agonist (µg/day)	Anti-leukotriene (mg/day)
1	62	F	96	0.96	2.7	No	Yes	Fluticasone propionate 500	No	No
2	26	M	102	0.75	0.78	No	Yes	Fluticasone propionate 250	No	No
3	51	M	90	0.72	1.76	No	ND	Budesonide 160	Formoterol 4.5	No
4	36	F	115	0.80	0.23	no	yes	fluticasone propionate 500	No	No
5	79	F	89	0.65	0.33	No	No	Fluticasone propionate 500	No	No
6	31	F	86	0.73	0.33	No	Yes	Fluticasone propionate 500	No	No
7	58	M	101	0.84	1.54	No	No	Budesonide 320	Formoterol 9	No
8	52	M	90	0.76	0.62	No	No	Fluticasone propionate 500	Salmeterol 50	No
9	32	M	91	0.74	3.8	No	Yes	Fluticasone propionate 500	No	No
10	50	F	94	0.77	0.17	No	No	Mometasone 200	No	No
11	36	M	104	0.78	2.15	No	Yes	Budesonide 400	No	10
12	27	F	96	0.72	0.4	No	Yes	Budesonide 400	No	No
13	26	F	115	0.91	2.3	Yes	No	Ciclesonide 160	No	No
14	66	M	84	0.74	2.5	No	Yes	Ciclesonide 160	No	No
15	17	F	84	0.88	0.05	No	Yes	Budesonide 400	No	10
16	55	M	99	0.76	3.5	No	No	Budesonide 200	No	0

FEV₁, forced expiratory volume in 1 s.**Table 2.** Clinical characteristics of patients with rhinitis

Patient No.	Age	Sex	FEV ₁ % predicted	FEV ₁ /VC ratio	Smoking	Atopy	Local nasal therapy (µg/day)
1	55	F	115	0.79	No	Yes	No
2	36	F	123	0.79	No	No	No
3	16	M	102	0.90	No	Yes	Mometasone 50
4	20	F	118	0.82	No	Yes	Mometasone 50
5	36	F	94	0.77	No	Yes	Mometasone 50
6	35	F	123	0.77	No	No	No
7	22	M	109	0.85	No	Yes	No
8	28	F	82	0.74	No	No	No
9	27	M	101	0.85	No	Yes	No
10	32	M	97	0.78	No	Yes	No
11	26	F	115	0.91	No	No	No
12	52	F	97	0.78	No	Yes	No
13	19	F	94	0.91	No	No	No
14	28	M	99	0.97	No	Yes	No
15	41	F	97	0.72	Yes	No	Fluticasone 50
16	21	M	101	0.88	No	Yes	Mometasone 50
17	29	M	81	0.70	Yes	Yes	No
18	17	M	81	0.81	No	Yes	No

FEV₁, forced expiratory volume in 1 s.

TNF-α represent a discrete population that is unique in its FL-3 intensity. The angiogenic cytokine capture beads were incubated with recombinant standards or test samples (sputum supernatant) with phycoerythrin-conjugated detection antibodies to form sandwich complexes. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (BD Biosciences). Data were acquired and analysed using Becton Dickinson Cytometric Bead Array CBA software, and the concentrations were extrapolated from the standard curves by plotting the recombinant

calibrator concentration against the FL-2 mean fluorescence intensity.

Statistical analyses

The distribution of data was calculated using the Shapiro–Wilk test. Because the data were not normally distributed, we performed a Kruskal–Wallis test and used Dunn's method to isolate groups that differed from one other. The strength of association between variables was obtained by

the Spearman rank-order method. Probability values of $P < 0.05$ were considered to be statistically significant. Analyses were performed with SPSS (version 10.0) and GraphPad Prism (version 5.1).

Results

The results of the counts of inflammatory cells in the induced sputum of asthmatic, rhinitis patients, and control subjects are presented in Table 3. The percentages of eosinophils in the induced sputum were significantly increased ($P < 0.0001$, Kruskal–Wallis test) in asthmatic patients compared with rhinitis and control subjects.

The concentrations of angiogenin in the induced sputum were significantly elevated ($P < 0.0001$, Kruskal–Wallis test) in both rhinitis (median, range 54.04, 40.5–62.02 ng/mL; $P < 0.001$, Dunn's method) and asthma (median, range 59.9, 42.7–65.10 ng/mL; $P < 0.001$, Dunn's method) patients compared with the healthy control group (median, range 13.5, 7.8–30.03 ng/mL) (Fig. 1a). Similarly, the concentrations of VEGF were also markedly elevated ($P = 0.0005$, Kruskal–Wallis test) in rhinitis (median, range 986.0, 577.8–1614 pg/mL; $P < 0.01$, Dunn's method) and asthmatic (median, range 1118, 482.2–1918 pg/mL; $P < 0.01$, Dunn's method) patients compared with controls (median, range 285.2, 155.9–602.1 pg/mL) (Fig. 1b). There was no significant difference ($P = 0.3$, Kruskal–Wallis test) in the concentrations of IL-8 between the study groups (median, range rhinitis: 1.6, 0.8–5.2 ng/mL; asthma: 0.94, 0.5–3.4 ng/mL; controls: 0.9, 0.3–2.3 ng/mL) (Fig. 1c). The concentrations of bFGF were low (typically less than the sensitivity rate of 2.9 pg/mL) in all study subjects. The concentrations of TNF- α in the sputum were also low; however, a significant peak ($P = 0.02$, Kruskal–Wallis test) was observed in the rhinitis group (median, range 15.5, <4.6–69.2 pg/mL; $P < 0.05$, Dunn's method) compared with healthy controls (median, range 3.9, <1.2–4.7 pg/mL) but not asthma patients (median, range 1.8, <1.2–32.2 pg/mL).

There was a significant correlation between angiogenin and VEGF concentrations ($R = 0.84$, $P < 0.0001$) as well as angiogenin or VEGF and the number of sputum lymphocytes ($R = 0.4$, $P = 0.003$) in our study subjects. There was no significant correlation between the other angiogenic

factors and the numbers of eosinophils, neutrophils, macrophages, lymphocytes, patient age, or pulmonary function results.

Discussion

Here, we have evaluated the concentrations of VEGF, angiogenin, IL-8, bFGF, and TNF- α in the airways of adults with rhinitis and patients with controlled asthma. VEGF and angiogenin levels were significantly higher in the induced sputum of both groups of patients with rhinitis and asthma than that of healthy subjects. These findings therefore suggest ongoing angiogenesis in patients with rhinitis without concomitant asthma and in corticosteroid-treated and well-managed asthmatic patients.

The primary point of our study was to evaluate angiogenic profiles in the induced sputum of patients with rhinitis. The results showed that angiogenin and VEGF concentrations were significantly higher in rhinitis patients than in healthy controls. Our data suggest that angiogenesis might be active in the lungs of patients with rhinitis without concomitant asthma. A similar increase of VEGF and its receptor was also observed in the nasal mucosa of seasonal AR patients [39, 40]. Very recently, Sohn et al. [41] demonstrated increased VEGF mRNA in patients with AR. In our study, we further confirmed these data at the protein level. Moreover, we also demonstrated significantly increased angiogenin levels that correlated with VEGF.

These results indicate that additional prospective work is needed to elucidate the role of these mediators in the pathogenesis of rhinitis and early development of asthma.

Previous studies based on immunocytochemical staining of bronchial biopsy tissue showed that VEGF and angiogenin were significantly increased in the airways of steroid-naïve asthmatic patients compared with control subjects [42]. Recently, they have demonstrated increased levels of VEGF and angiogenin in the induced sputum of asthmatic steroid-naïve children during asthma exacerbation. [43]. The VEGF concentrations found in the induced sputum of mild, steroid-naïve asthmatics were also associated with the degree of airway narrowing [44]. We would like to emphasize our finding of elevated levels

Table 3. Cell counts in the induced sputum of control, asthma, and rhinitis subjects

	Controls ($n = 15$)	Rhinitis ($n = 18$)	Asthma ($n = 16$)
Total cell number/ μ L of sputum	2520 (1305–6480)	3353 (570–6300)	1350 (600–1845)
Neutrophils (%)	53 (27–69)	59 (21.8–89.3)	48.5 (15.5–72.8)
Eosinophils (%)	1 (0–1)	1 (0–2)	4.5 (3–28.3)*
Macrophages (%)	32 (25–67)	35.5 (7–66.8)	35.5 (11.3–65.8)
Lymphocytes (%)	4 (2–5)	4 (2.8–6)	4.5 (2–7.5)

*Significantly different from the values for control subjects and patients with rhinitis ($P < 0.0001$; Kruskal–Wallis test).

Data are presented as medians, with interquartile ranges in parentheses.

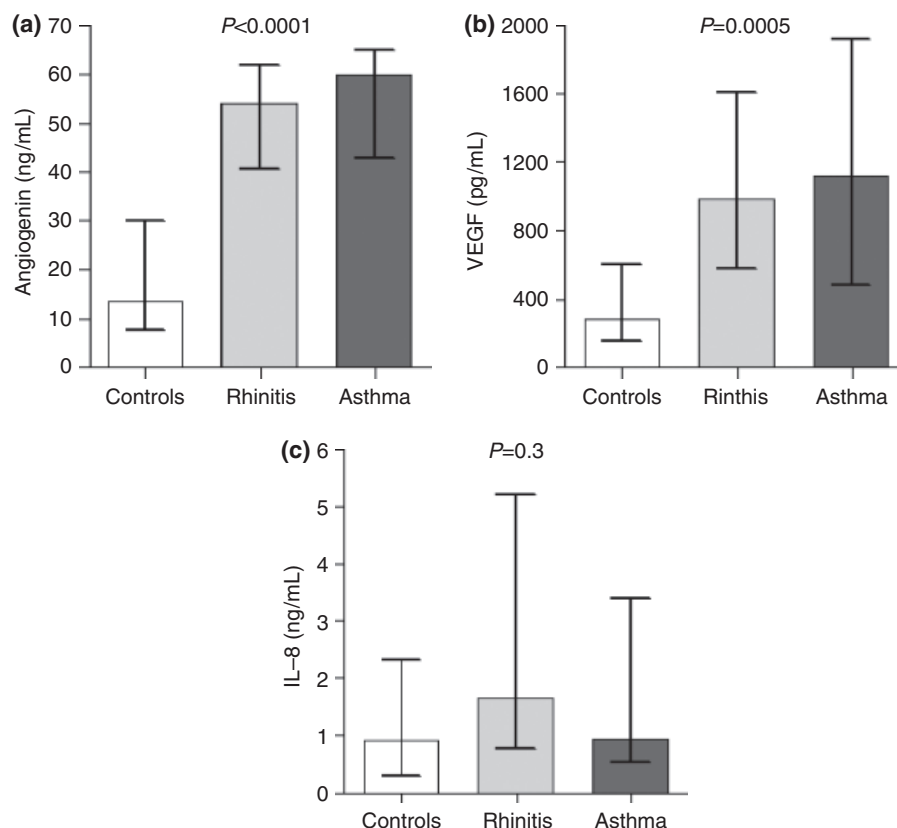


Fig. 1. Angiogenin (a) and VEGF (b) concentrations were significantly higher ($P \leq 0.0005$; Kruskal–Wallis test) in the induced sputum of subjects with rhinitis ($n = 18$) and controlled asthma ($n = 16$) than that of healthy controls ($n = 15$). There was no difference ($P = 0.3$; Kruskal–Wallis test) in terms of IL-8 (c) concentrations. The bars indicate the median values and inter-quartile ranges for each group. VEGF, vascular endothelial growth factor.

of angiogenic factors in the sputum of patients who were well controlled on their inhaled corticosteroid therapy; in contrast to previous studies, we did not include steroid-naïve patients in this study [42–44]. These results suggest that angiogenesis still occurs in the lungs of well-controlled asthmatic patients. Because our study did not include a steroid-naïve group of patients with asthma, we cannot make a comparison between the levels of angiogenic factors in treated and steroid-naïve patients. However, previously published data have demonstrated the anti-angiogenic effectiveness of inhaled corticosteroids and long-acting β -agonists in asthma [45, 46]. We also found a significant positive correlation between the levels of angiogenin and VEGF. Therefore, angiogenin and VEGF might play coordinated and synergistic roles in asthma angiogenic remodelling. Our results support the recent report by Simcock et al., who highlighted the importance of VEGF in the pathogenesis of asthma. With selective immunodepletion of VEGF in the bronchoalveolar lavage fluid (BALF) from asthmatic patients, these authors demonstrated abolished pro-angiogenic capacity and confirmed that the pro-angiogenic capacity of BALF from asthmatics resides predominantly in VEGF from the asthmatic airway. This finding suggests that VEGF is

present in the asthmatic airway and is necessary for asthma-induced angiogenesis *in vitro* [13].

We did not find any significant difference in IL-8, TNF- α , or bFGF levels in the induced sputum of asthmatic patients in comparison with that from either the control group or the group of patients with rhinitis. In previous studies, IL-8 was found in elevated levels in the induced sputum of patients with severe asthma [17–21]. We may therefore conclude that our result is in concordance with previous reports [17–21], because we have studied IL-8 in the airways of well-controlled and not severe asthmatics.

In contrast to the results of our study, previous studies have considered bFGF to be an important mediator in asthma airway remodelling. Increased bFGF levels were demonstrated in bronchial biopsy tissue and BALF following allergen challenge [42, 47]. One possible reason for this discrepancy could be related to the fact that we have included well-controlled and treated asthmatics in our studies; previous studies have included only steroid-naïve patients.

We did not demonstrate that TNF- α is an important mediator of angiogenesis in our asthma patients. However, we showed that TNF- α levels are significantly elevated in the induced sputum of patients with rhinitis

without concomitant asthma in comparison with that from the control group. Berry *et al.* [48] have demonstrated an increased expression of membrane-bound TNF- α by peripheral blood monocytes in refractory asthma and suggested that TNF- α has a number of properties that may be relevant to refractory asthma. One possible reason for this discrepancy is again related to the fact that our patients had controlled asthma and not refractory asthma as in previous studies.

In summary, we showed markedly increased VEGF and angiogenin concentrations in the airways of patients with rhinitis without concomitant asthma. Furthermore, we also demonstrated significantly elevated VEGF and angiogenin concentrations in corticosteroid-treated and well-controlled asthmatic patients. Our study supports the importance of airway angiogenesis in the pathogenesis of rhinitis. It also supports continued investigation of VEGF and angiogenin for the development of new treatments that affect airway remodelling.

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