Increased expression of angiogenic markers in patients with seasonal allergic rhinitis

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ABSTRACT. *Background.* **Increased vascularity due to neo-angiogenesis is an essential part of airway remodelling. Vascular endothelial growth factor (VEGF), CD34 and von Willebrand's factor (FvW) are known angiogenic markers. Angiogenesis and airway remodelling has been documented in asthma but not in allergic rhinitis. Objective: We aimed to investigate the presence of increased angiogenesis and its relation to angiogenic molecules, namely VEGF, CD34 and FvW, in endothelial cells of nasal mucosa in patients with seasonal allergic rhinitis (SAR), using three different immunohistochemical analysis methods, namely HSCORE, microvessel density (MVD) and vascular surface density (VSD). The findings in allergic rhinitis were compared with the findings in nasal septal deviation (NSD), which is not associated with increased angiogenesis. *Methods.* Twenty patients with symptomatic SAR, who were not under treatment, were enrolled in the study. Ten patients with NSD, who needed surgical therapy, served as the control group. Demographic characteristics did not differ between the two groups. Inferior turbinate biopsy was obtained from SAR patients and control patients, under local anaesthesia and during surgery respectively. All biopsies were evaluated for angiogenesis on the basis of VEGF, CD34 and FvW by two blinded histologists using three immunohistochemical analysis methods (HSCORE, MVD and VSD). *Results.* HSCORE, estimated on the basis of each staining technique, showed statistically significant differences among the two groups (p=0.002; p=0.045; p=0.016, respectively). Anti-CD34 and anti-VEGF showed higher MVD values in SAR when compared to the controls (p=0.038; p=0,009, respectively). No statistically significant difference was found in Anti-FvW-based MVD between SAR patients and controls (p=0.071). The measurements of VSD for FvW and VEGF from nasal biopsy specimens displayed a statistically significant difference between the two groups (p=0.004; p=0.0001, respectively). However, measurement of VSD for CD-34 was not significantly different between the groups (p=0.086). On the other hand, morphometric data obtained by all three methods did not correlated. *Conclusion.* There are a few studies that have investigated the essential role of angiogenesis in the pathogenesis of allergic rhinitis. We conclude that, increased angiogenesis may be as prominent in patients with allergic rhinitis as in patients with non-allergic nasal pathologies and may play an important role in the remodelling of nasal mucosa of subjects with SAR.**

Keywords: allergic rhinitis, nasal septal deviation, angiogenesis, remodelling

INTRODUCTION

Allergic rhinitis is clinically defined as a symptomatic disease of the nasal mucosa caused by an IgE-mediated, allergic inflammation [1]. Allergic inflammation is mediated by several factors such as histamine, prostaglandins, leukotriens, and another inflammatory mediator namely vascular endothelial factor (VEGF) [1]. Additionally, allergic rhinitis is associated with sinusitis and other co-morbities such as asthma [1]. Airway remodelling, which results from persistent inflammation in the bronchial wall, associated with the production of proinflammatory cytokines and growth factors, is an essential element of asthma

pathogenesis [2-5]. Increased vascularity due to angiogenesis is an important part of remodelling [6]. Angiogenesis, the growth and proliferation of new blood vessels, is important in a variety of pathophysiological processes, such as tumour growth, rheumatoid arthritis and asthma [7-10]. However, there are few studies which have investigated the role of angiogenesis in allergic rhinitis.

Vascular endothelial growth factor, which was formerly known as vascular permeability factor, is responsible for increased capillary permeability, a potent inducer of endothelial cell growth and angiogenesis [8, 11-13]. Because angiogenesis and increased vascular permeability are char-

acteristic features of wound healing, VEGF may play an important role in nasal mucosal inflammation in allergic rhinitis. Additionally, it has been well documented that VEGF is increased in the nasal mucosa of patients with allergic rhinitis, as a result of the increase in nasal vascular permeability and congestion [14]. Additionally, one study has demonstrated an increase in angiogenic factors, such as platelet-derived endothelial cell growth factor, in allergic rhinitis [15]. Angiogenesis is mainly evaluated on the basis of microvessel density (MVD), which can be measured using various endothelial markers, such as von Willebrand's factor (FvW) and CD34 [16, 17]. Additionally, vascular surface density (VSD) is a stereological technique for the assessment of angiogenesis, which can be measured by immunostaining of these antigens [18]. These antigens that are demonstrated with immunostaining, can be measured using the HSCORE technique.

In our study, we aimed to investigate the levels of VEGF, CD34 and FvW expression in the nasal mucosa of patients with seasonal allergic rhinitis (SAR), and other nasal diseases such as nasal septal deviation (NSD), which is not associated with mucosal pathology.

PATIENTS AND METHODS

Patients

Twenty (12 females, eight males) patients with SAR were included in this study, along with 10 (six females, four males) patients with NSD as the non-allergic control group. Ethical concerns prohibited the enrolment of a healthy control group in the study.

In the patients with SAR, the mean age was 26.52 ± 5.91 (age range between 19 and 37) years. Diagnosis of seasonal allergic rhinitis was based on history, physical examination and laboratory findings. Allergic sensitization was demonstrated by the skin prick test. All patients with SAR had positive skin prick test reactivity to grass/cereal allergen mixture (*Hulcus lanatus, Lolium perenne, Fectuca pratensis, Phleum pratense, Poa pratensis, Dactylis glomerata, Hordeum vulgare, Avena sativa, Secale cerela, Triticum sativum*) (Allergopharma Ltd, Reinbek, Germany). Skin prick tests were performed according to the EAACI guidelines [19].

The mean age of the controls was 24.45 ± 7.24 (age range between 18 and 39) years. These non-allergic control patients were diagnosed by an otolaryngologist, based on history, physical examination, accompanied by rhinoscopy and laboratory findings. Allergen skin prick test was negative in all non-allergic, control group individuals.

Thiny inferior turbinate biopsies were obtained from SAR patients using a cup forceps device under topical anaesthesia, followed by fixation with 10 % formalin in phosphate-buffered saline (PBS) for a maximum duration of 24 hours. They were embedded in paraffin using a routine embedding procedure. The same procedure was performed on the control group during septoplasty by the same otolaryngologist.

Informed consent for the described procedures was obtained from all patients. Approval for the study was given by the ethics committee of our hospital.

Immunohistochemistry

Antibodies

Monoclonal mouse antibodies were used to demonstrate VEGF (Santa Cruz Biotechnology, sc-7269, California, USA, diluted to a 1:200 ratio), FvW (Dako, Mo-616, Glostrup, Denmark, diluted to a 1:100 ratio) and CD34 (Dako, Class II: M-7165, Glostrup, Denmark, diluted to a 1:100 ratio).

Immunohistochemical technique

In all cases, 5 μ-thick, paraffin-embedded sections of formalin-fixed tissues were used. Sections were deparaffinated and dehydrated through a series of graded ethanol solutions, then incubated in distilled water containing 0.3% H_2O_2 to inhibit endogenous peroxidase activity. Sections were stained with primary antibodies: anti-VEGF, anti-FvW and anti-CD34 for 18 h. After washing, the secondary antibody (biotinylated goat IgG anti-rabbit/mouse IgG) was applied for 30 min, followed by three washes in PBS. The streptavidin-peroxidase complex (Universal Dako LSAB 2 System-K0675, Glostrup, Denmark) was added for 30 min fallowed by three washes in PBS. Slides were counter-stained with Mayer's haematoxylin, dehydrated, and cleared, and after the application of a cover-slip, were analysed using a BX 40, light microscope (Olympus, Tokyo, Japan). The presence of a brown precipitate indicated positive findings for the primary antibody. Microvessels were defined as any brown-stained, endothelial cell or cell cluster that was clearly separated from adjacent microvessels, glandular cells, or connective tissue components [20]. Serial sections were examined, and staining patterns were compared. Two observers (KO, GG), who were blinded to the clinical information regarding the nasal inferior turbinate samples, evaluated the staining scores independently.

Immunohistochemical analysis
– Measurement of HSCORE

Nasal mucosal endothelial cell staining intensity was graded semi-quantitatively and the HSCORE was calculated using the following equation: HSCORE = Σ Pi (i+1), where i = intensity of staining with a value of 1, 2 or 3, (weak, moderate, or strong, respectively) and Pi being the percentage of stained endothelial cells for each intensity, varying from 0% to 100%.
– Measurement of MVD

Microvessel density was assessed by light microscopy based on the criteria of Weidner *et al.* [20]. In each section, the five most vascularized areas were selected and analyzed at ×100 magnification (0.192 mm^2 per field). Computer images (jpeg files) were used to perform manual counts of stained microvessels, which were marked after counting to prevent duplicate counts. As a result, MVD was expressed as the number of microvessels per mm^2.
– Measurement of VSD

In all samples, vWF-, CD34- and VEGF-labelled vessels were counted for the assessment of VSD. Slides were analyzed at ×200 magnification to identify the areas of vascularity near the surface epithelium for VSD measurement. Five fields in these areas, on each slide, were randomly selected for computer images (jpeg files). The unbiased stereology method was used to demonstrate the
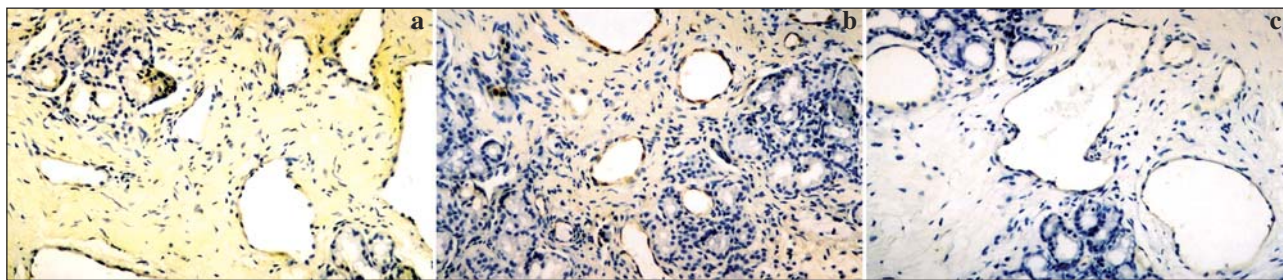
Figure 1
Photomicrograph of nasal mucosal biopsy of patients with seasonal allergic rhinitis, demonstrating anti-von Willebrand factor (a), anti-CD34 (b), anti-VEGF (c). Original magnification × 200.

estimation of VSD [18]. A cycloidal test system, with its minor axis parallel to the vertical direction, was randomly translated to the *x* and *y* directions on the images. The test system has a known length of cycloid per point (1/p). In order to estimate VSD, we needed to make two counts on this image; first, the number of intersections between the cycloid lines and the boundary of interest (I); and second, the number of points that landed within the reference space (P). Vascular surface density was then calculated from the formula: $VSD = 2 \times \Sigma I / (1/p) \times \Sigma P$.

Statistical analysis

The average of the results from the two investigators was used for statistical analysis. Morphometric data for HSCORE, MVD and VSD were expressed as mean (± SD) and analyzed by the Mann-Whitney U test. Correlation between three groups of morphometric data was evaluated using the Spearman's correlation test. *P* values less than 0.05 were accepted as statistically significant.

RESULTS

All demographic characteristics of patients with SAR and the controls were similar.

Light microscopic examination revealed that the nasal mucosa was composed of pseudostratified, prismatic epithelium and lamina propria, the latter being rich in glandular structures and blood vessels. Immunohistochemical evaluation of the nasal mucosa of patients with SAR revealed an abundance of blood vessels. Intense staining of the endothelial cells of these vessels for anti-VEGF was not observed in the stroma *(figure 1a)*. Staining of the endothelial and stromal cells in the control group was much weaker *(figure 2a)*. Anti-CD34 labelling also dem-

onstrated an abundance of blood vessels in the nasal biopsy specimens of SAR patients. The endothelial cells and the stromal cells of the vessels displayed CD34 immunoreactivity, while the glandular cells did not express CD34 *(figure 1b)*. A similar staining pattern was also observed in the control group *(figure 2b)*. Staining with anti-FvW demonstrated an intense staining pattern in vessel endothelium, while stromal staining was not as intense in SAR patients or the control group. Moreover, it was seen that glandular cells did not express FvW *(figure 1c and figure 2c)*.

These molecules were evaluated in the nasal mucosa using the HSCORE, MVD and VSD methods. HSCORE, MVD and VSD values, estimated on the basis of VEGF-, CD34- and FvW- labelling in patients with SAR and in controls, are shown in *figures 3a, b and c*.

Results of HSCORE

Anti-VEGF, Anti-CD34 and Anti-FvW showed higher HSCORE values in patients with SAR when compared to the control group (274.70 ± 57.38 *versus* 218.80 ± 36.84 *P*=0.016; 241.80 ± 56.70 *versus* 191.20 ± 43.93 *P*=0.045; 188.70 ± 32.11 *versus* 150.50 ± 22.41 *P*=0.002, respectively).

Results of MVD

The VEGF labeling showed higher MVD values in SAR than in the control group (31.21 ± 7.02 *versus* 24.60 ± 3.90 P=0.009). Similarly, measurement of MVD for CD34 was significantly higher in patients with SAR as compared to the control group (37.32 ± 8.23 *versus* 30.95 ± 7.00 *P*=0.038). Anti-FvW evaluation did not demonstrate any differences (32.33 ± 7.61 *versus* 27.62 ± 5.04 *P*=0.071).
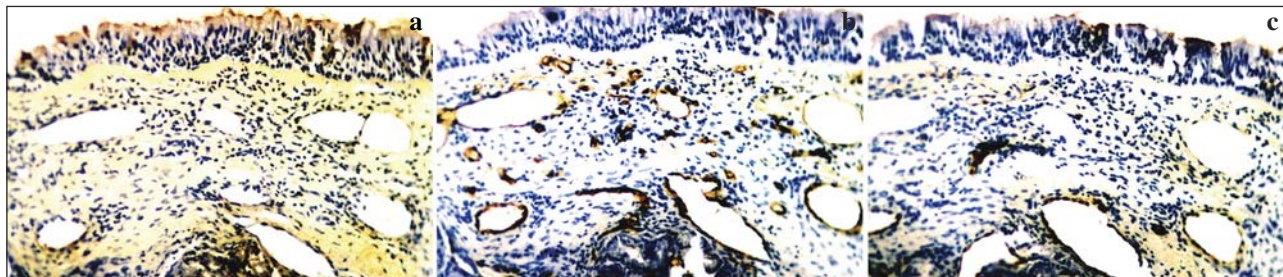
Figure 2
Photomicrograph of nasal mucosal biopsy of patients with nasal septal deviation, demonstrating anti-von Willebrand factor (a), anti-CD34 (b), anti-VEGF (c). Original magnification × 200.
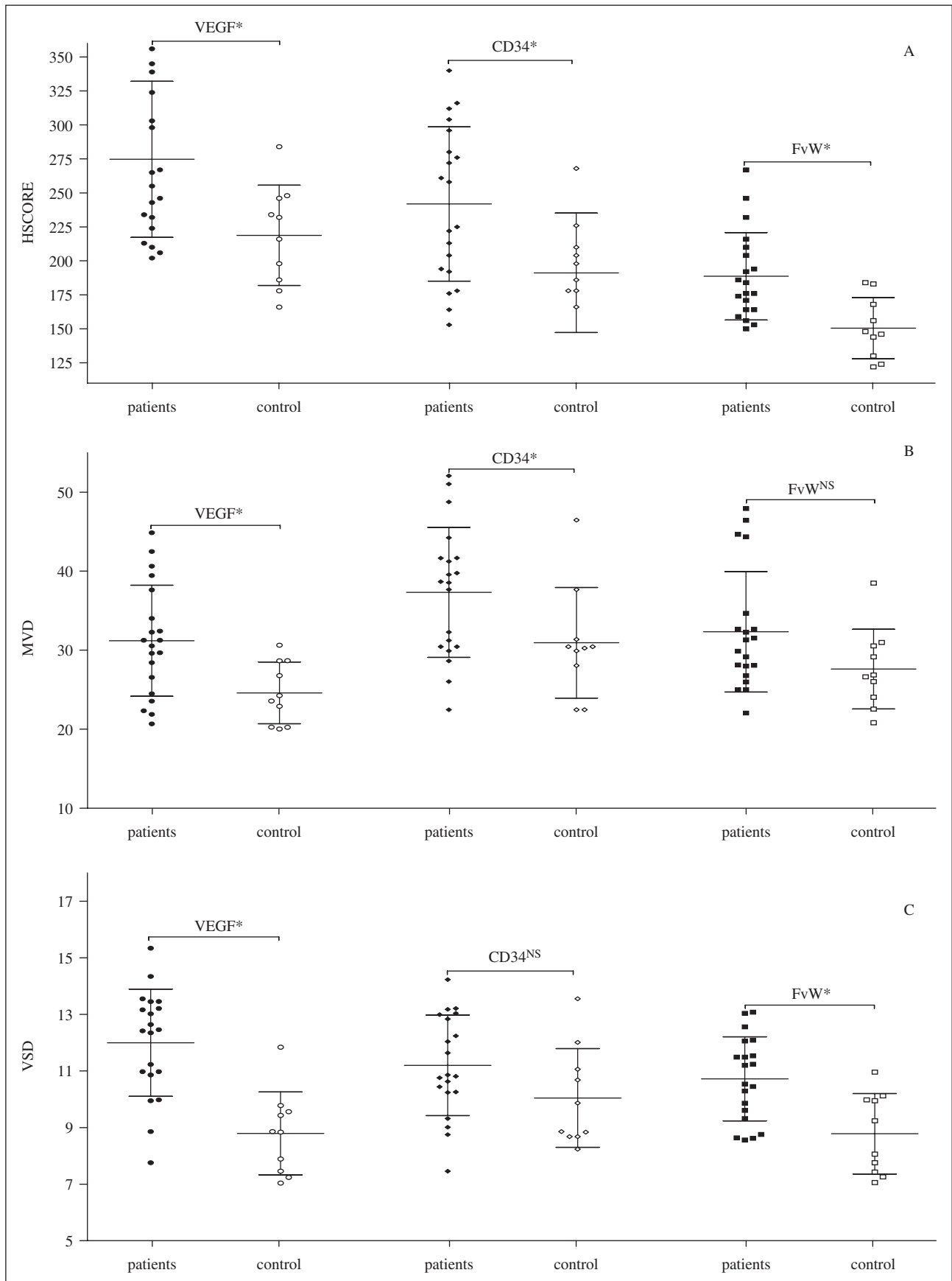
Figure 3

The HSOCRE values for VEGF, CD34 and FvW in patients and controls (a). The MVD values for VEGF, CD34 and FvW in patients and controls (b). The VSD values for VEGF, CD34 and FvW in patients and controls (c). *: See text for *P* values. [NS]: Non-significant.

Results of VSD

The measurements of VSD for VEFG and FvW revealed a statistically significant difference between the two groups (12.00 ± 1.89 *versus* 8.79 ± 1.47 *P*=0.0001; 10.72 ± 1.48 *versus* 8.78 ± 1.42 *P*=0.004, respectively). The CD-34 labeling did not show a difference (11.20 ± 1.78 *versus* 10.05 ± 1.75 *P*=0.086).

It was demonstrated that there was no correlation between the morphometric data (HSCORE, MVD and VSD) evaluated on the basis of FvW, CD34 and VEGF.

DISCUSSION

Allergic rhinitis, which is a chronic disease characterized by allergic inflammation, is associated with co-morbidities, especially allergic asthma [1]. Studies on asthmatic patients have demonstrated that bronchial airway remodelling begins with vascular changes, increased angiogenesis or neo-angiogenesis [3-6, 9, 10]. Thus, detection of increased angiogenesis may be used as an indicator of remodelling. However, the number of studies evaluating remodelling in the nasal mucosa of patients with allergic rhinitis combined with allergic asthma is limited. Nevertheless, a few other studies have demonstrated the presence of angiogenic factors in certain nasal inflammatory pathologies such as allergic rhinitis and nasal polyps [14, 15, 21]. On the other hand, there are many molecular-based studies which have demonstrated the expression of growth factors such as VEGF in the pathogenesis of inflammatory process in nasal polyps, leading to angiogenesis and remodelling [22, 23]. However, inflammation or increased angiogenesis in upper airway mucosa has not been detected in the non-inflammatory nasal pathologies such as NSD, which seems to be an anatomical pathology.

We have documented a significantly increased expression of the three antigens that indicate the presence of angiogenesis and/or neo-angiogenesis in SAR patients, as compared to control subjects with NSD. Increased angiogenesis and remodelling, secondary to allergic inflammation in nasal mucosa of SAR patients, can be demonstrated by immunohistochemical techniques. Previous studies have shown increased angiogenesis in patients with allergic rhinitis when compared normal controls, and it has been stated that this plays a role in inflammatory pathogenesis [14, 15, 24].

As mentioned above, we have shown immunohistochemically that patients with SAR, express markers that are indicators of angiogenesis more intensely than control subjects with NSD who display increased angiogenesis in their nasal mucosa. HSCORE, which evaluates immunohistochemically the vascular structures in the microscopic field, has demonstrated that expression of FvW, CD34 and VEGF is significantly more intense in patients with SAR. Therefore, it may be concluded that, the significantly increased levels of angiogenesis, as detected by HSCORE in these patients, as compared to NSD patients, may indicate the presence of neo-angiogenesis and remodelling in SAR. Moreover, the VSD method that evaluates the density of the vascular area microscopically, revealed a significant, immunohistochemical difference in FvW and VEGF between patients with SAR and the control group. There are no previous studies in the medical literature that have evaluated angiogenesis in patients with SAR or any other nasal inflammatory disease using the VSD method. Additionally, evaluation with the MVD method also detected a difference in expressions of CD34 and VEGF between the groups, and the results demonstrated a significantly increased expression in the SAR group. There are many previous studies that have evaluated angiogenesis in various pathological conditions, particularly uterine diseases, using the MVD method [16, 17, 25]. Moreover, there are many review articles which indicate that MVD is the best method for evaluating angiogenesis [26, 27]. Poncelet *et al.* in a study that evaluated angiogenesis in leiomyomas using FvW, CD34 and VEGF, have concluded that CD34 is a better angiogenic marker [17]. Our study has demonstrated an increased MVD based on CD34, a superior marker of neo-angiogenesis, in patients with SAR compared to NSD patients, and this might indicate that the extent of angiogenesis is greater in patients with SAR. VEGF has been observed to increase significantly regardless of the immunohistochemical analysis method used. VEGF, which was formerly identified for its ability to induce vascular permeability and stimulate endothelial cell growth, is now recognized as a key factor required for growth of tumours, and is involved in many other diseases, such as diabetes, arthritis, atherosclerosis and ischemic heart disease [8, 11-13, 28]. Furthermore, in the light of our study, increased VEGF detection by all immunohistochemical methods, suggests that VEGF plays an important role in the angiogenic process of allergic rhinitis. The absence of correlation between morphometric data shows that all three techniques evaluate angiogenesis and remodelling independently and from a different perspective.

In conclusion, although we have not evaluated other markers of remodelling, our observation suggests that the presence of angiogenesis and related remodelling cannot be denied in patients with SAR despite a certain amount of controversy. Proving the presence of increased angiogenesis, an important part of remodelling and other chronic inflammatory changes in the nasal mucosa, may improve treatment choices, and follow-up in subjects with SAR.

REFERENCES

1. Bousquet J, Van Cauwenberge P, Khaltaev N. Allergic rhinitis and its impact on asthma. *J Allergy Clin Immunol* 2001; 108(Supp): 148.

2. Wilson JW, Li X. The measurement of reticular basement membrane and submucosal collagen in the asthmatic airway. *Clin Exp Allergy* 1997; 27: 363.

3. Bousquet J, Vignola AM, Chanez P, Campbell AM, Bonsignore G, Michel FB. Airways remodelling in asthma: no doubt, no more? *Int Arch Allergy Immunol* 1995; 107: 211.

4. Wilson JW, Li X. Inflammation and cytokines in airway wall remodelling. In: A G S, ed. *Airway wall remodelling in asthma. Boca Raton*. Florida. USA: CRC Press, 1997: 65.

5. Redington AE, Howarlh PH. Airway wall remodelling in asthma. *Thorax* 1997; 52: 310; [editorial].

6. Wilson JW, Bamford TL. Assessing the evidence for remodelling of the airway in asthma. *Pulm Pharmacol Ther* 2001; 14: 229.

7. Klagsbrun M, D'Amore PA. Regulators of angiogenesis. *Annu Rev Physiol* 1991; 53: 217.

8. Folkman J, Shing Y. Angiogenesis. *J Biol Chem* 1992; 267: 10931.

9. Li X, Wilson JW. Increased vascularity of the bronchial mucosa in mild asthma. *Am J Respir Crit Care Med* 1997; 156: 229.

10. Hoshino M, Nakamura Y, Hamid QA. Gene expression of vascular endothelial growth factor and its receptors and angiogenesis in bronchial asthma. *J Allergy Clin Immunol* 2001; 107: 1034.

11. Ferrara N, Houck K, Jakeman L, Winer J, Leung DW. The vascular endothelial growth factor family of polypeptides. *J Cell Biochem* 1991; 47: 211.

12. Leung DW, Cachianes G, Kuang W-J, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 1989; 246: 1306.

13. Keck PJ, Hauser SD, Krivi G, *et al*. Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science* 1989; 266: 1309.

14. Benson M, Carlsson B, Carlsson LMS, Wennergren G, Cardell LO. Increased expression of vascular endothelial growth factor- in seasonal allergic rhinitis. *Cytokine* 2002; 20: 268.

15. Mori S, Fujieda S, Sunaga H, Fox SB, Saito H. Expression of platelet-derived endothelial cell growth factor and vascularity in the nasal mucosa from allergic rhinitis. *Clin Exp Allergy* 2000; 30: 1637.

16. Loggini B, Boldrini L, Gisfredi S, *et al*. CD34 microvessel density and VEGF expression in basal and squamous cell carcinoma. *Pathol Res Pract* 2003; 199: 705.

17. Poncelet C, Madelenat P, Feldmann G, Walker F, Darai E. Expression of von Willebrand's factor, CD34, CD31, and vascular endothelial growth factor in uterine leiomyomas. *Fertil Steril* 2002; 78: 581.

18. Howard CV, Reed MG. *Unbiased Stereology: Three Dimensional Measurement in Microscopy*. Oxford: Bios Scientific Publishers Ltd, 1998; 115.

19. Dreborg S, Frew A. EAACI Subcommittee on Skin Tests. Position paper. Allergen standardization and skin tests. *Allergy* 1993; 48(suppl 14): 49.

20. Weidner N, Carroll PR, Flax J, Blumenfeld W, Folkman J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am J Pathol* 1993; 143: 401.

21. Wittekindt C, Hess A, Bloch W, Sultanie S, Michel O. Immunohistochemical expression of VEGF and VEGF receptors in nasal polyps as compared to normal turbinate mucosa. *Eur Arch Otorhinolaryngol* 2002; 259: 294.

22. Coste A, Brugel L, Maitre B, *et al*. Inflammatory cells as well as epithelial cells in nasal polyps express vascular endothelial growth factor. *Eur Respir J* 2000; 15: 367.

23. Ito A, Hirota S, Mizuno H, *et al*. Expression of vascular permeability factor (VPF/VEGF) messenger RNA by plasma cells: possible involvement in the development of oedema in chronic inflammation. *Pathol Int* 1995; 45: 715.

24. Svensson C, Andersson M, Greiff L, Alkner U, Persson CG. Exudative hyperresponsiveness of the airway microcirculation in seasonal allergic rhinitis. *Clin Exp Allergy* 1995; 25: 942.

25. Chen CA, Cheng WF, Lee CN, *et al*. Cytosol vascular endothelial growth factor in endometrial carcinoma: correlation with disease-free survival. *Gynecol Oncol* 2001; 80: 207.

26. Teo NB, Shoker BS, Martin L, Sloane JP, Holcombe C. Angiogenesis in pre-invasive cancers. *Anticancer Res* 2002; 22: 2061.

27. Hannen EJ, Riediger D. The quantification of angiogenesis in relation to metastasis in oral cancer: a review. *Int J Oral Maxillofac Surg* 2004; 33: 2.

28. Verheul HM, Pinedo HM. Vascular endothelial growth factor and its inhibitors. *Drugs Today (Barc)* 2003; 39(Suppl C): 81.