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Expression of Vascular Endothelial Growth Factor and Fibronectin in Nasal Polyps: Effects of Topical Corticosteroid

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배경: 비용종은 염증세포 침윤, 조직의 부종 및 개형 등의 소견을 보이지만 이의 발생 및 성장의 정확한 기전은 아직 알려져 있지 않다.
목적: 혈관내피성장인자(vascular endothelial growth factor, VEGF)와 섬유결합소(fibronectin: Fn)는 손상된 조직의 치유과정에서 중요한 역할을 하는 인자들로 알려져 있다. 본 연구는 비용종의 조직형에 따른 VEGF 및 Fn의 발현 양상 및 이들에 대한 스테로이드의 효과를 관찰하였다.
방법: 비폐색의 개선을 위해 비용종 절제술을 받은 환자 59명의 비용종 조직을 이용하여 이들의 조직학적 소견과 면역조직화학염색을 통한 VEGF와 Fn의 발현 양상을 평가하였다. 비용종 환자들 중 41명은 스테로이드를 투여 받은 적이 없었으며, 18명은 수술 전에 장기간의 국소 스테로이드 요법을 받았다(Nasacort AQ, Fisons Ltd., UK: 하루 220μg을 평균 40일간 투여). 대조군으로는 성형 목적으로 비수술을 받은 9명의 환자에서 얻은 정상적인 비중격 점막 조직을 함께 조사하였다.
결과: 비용종 조직은 정상 비점막에 비해 VEGF와 Fn의 발현이 증가되어 있었다. VEGF 및 Fn의 발현 정도는 종창형, 선상 및 낭상형, 섬유형의 순이었으며, VEGF와 Fn의 발현 사이에는 상관관계가 있었다. 스테로이드를 사용하지 않은 군에 비해 사용한 군의 조직은 부종이 감소한 반면에 더 섬유화된 소견을 보였으며 VEGF와 Fn의 발현이 현저히 감소하였다.
결론: VEGF 및 Fn이 혈관투과성을 항진시키고 조직의 염증 및 개형에 관여함을 고려할 때, 비용종 조직에서의 VEGF 및 Fn의 생성 증가는 비용종의 성장과정에서 중요한 역할을 할 것으로 추측된다. 또한 국소 스테로이드 투여로 인한 VEGF 및 Fn의 생성 감소는 이 약제가 비용종의 치료제로서 유효한 기전의 하나로 생각된다. **(Korean J Asthma Allergy Clin Immunol 2005;25:97-103)**

Key words: Vascular endothelial growth factor, Fibronectin, Nasal polyp, Corticosteroid

INTRODUCTION

Nasal polyp (NP), a smooth and pale grape-like pedunculating mass attached to the paranasal sinus, is characterized by increased exudation from vessels, edema of the lamina propria, infiltration of inflammatory cells, and thickening of basement membrane.[1-5] Various cytokines or chemokines, and extracellular matrix (ECM) molecules appear to be related with these inflammatory and remodeling process of NP, but the exact pathogenesis of NP is still unclear.

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, and fibronectin (Fn) have been considered to play a role in the pathogenesis of NP.[6-8] VEGF may be closely involved in the formation of edema in NP by increasing vascular permeability and angiogenesis. Considering the role of Fn in the process of tissue morphogenesis and remodeling,[9] in particular fibrosis, Fn may also play an important role in the shaping of NP. In this study, we investigated the degree and distribution of VEGF and Fn expression according to the histologic types of NP, and examined the effect of topical steroid on their expressions.

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MATERIALS AND METHODS

1. Subjects

NP tissues from 59 patients with clinical records, histological slides and paraffin blocks were selected among patients who received nasal polypectomy to improve nasal obstruction from January 1999 to March 2001. Normal nasal mucosa (NNM) tissues of septum, obtained from 9 patients who had nasal plastic surgery, were used as controls. The Institutional Review Board approved retrospective review of medical records from patients. Among the 59 nasal polyposis patients, 41 patients had never

received steroid therapy either systemically or locally (Group I) and 18 patients had a history of local steroid therapy just prior to surgery (Group II). The average ages of Group I and Group II were 38.1±16.8 and 45.2±15.7 respectively, and the male to female ratio was 25 : 16 and 14 : 4. The steroid used in Group II was Triamcinolone acetonide nasal solution (Nasacort AQ, Fisons Ltd., UK) with a 220μg/day dosage for a duration of 40± 27 days before polypectomy. Most patients with NP had clinical or radiological evidence of sinusitis (fungal sinusitis in 3 cases), and received various kinds of antibiotics.

2. Light microscopic study

NPs were classified into three histologic types on hematoxylin-eosin (H&E) stain, according to the classification proposed by Kakoi and Hiraide[10]: (1) the edematous type, in which interstitium was composed mainly of edematous connective tissue and some glands were often seen without cyst formation; (2) the glandular and cystic type, in which mixed glands and cysts formation was invariably seen; and (3) the fibrous type, in which proliferation of fibroblasts and collagen fibers were prominent. When polyps revealed overlapping features of more than two types, portions of a more predominant histologic type were chosen. Change in epithelium and the degree of infiltration of eosinophils and plasma cells were evaluated by H&E stain. Masson's trichrome stain was used for the evaluation of fibrotic changes, and toluidine blue stain was used for the mast cells. The degree of infiltration of eosinophils and plasma cells was evaluated semi-quantitatively as follows: it was recorded as 0 when there was no infiltration, as 1+ when rarely scattered in the field, as 2+ when partially infiltrated and the area occupied less than 50% of submucosal layer, and as 3+ when there was massive infiltration occupying more than 50% of the submucosal area with cellular infiltration making distinguishing the individual cells impossible.

3. Immunohistochemical staining

The paraffin embedded tissues were cut in serial sections of 3μm thickness and placed onto slides coated with silane. These sections were deparaffinized, and then rehydrated. To block endogenous peroxidase activity in the biopsy specimen, sections were treated with 0.3% hydrogen peroxide in methanol at room temperature. For VEGF staining, the slides were boiled in a microwave oven and sunken in citric acid solution for 10 minutes, then put in room temperature for 20 minutes. For Fn staining, the specimens were treated with 0.1% trypsin (Sigma Chemical Co. St. Louis, MO) in 0.1% calcium chloride solution for 30 minutes at 37°C and then with 0.05% 4-chloro-1-naphthol solution in 0.05 mol/L Tris-HCl buffer (pH 7.6). The sections were incubated overnight at 4°C with the primary antibody: monoclonal antibody to VEGF (1 : 100 dilution; MS-1467, Neomarkers, USA) and monoclonal antibody to Fn (1 : 200 dilution; NCL-FIB, Novocastra, UK). After incubation with the primary antibodies, immunodetection was performed with biotinylated anti-mouse serum with a streptavidine/peroxidase (Dako-LSAB kit, K690, USA) for 15 minutes, then followed by applying DAB (3,3"-diaminobenzidine tetrahydrochloride) for the final color reaction. The sections were counterstained with Mayer's hematoxylin for 5 minutes. Saturation of non-specific antigenic sites with bovine serum albumin with omission of the primary antibody was used as negative control.

4. Evaluation of the expression of VEGF and Fn

Two pathologists who were blinded to patients' data examined all the samples. The expression of VEGF and Fn was classified as positive when the cytoplasm was stained brown. The expression of VEGF was measured using a semi-quantitative scale on surface epithelium, glandular epithelium, and inflammatory cells. It was recorded as 0 when the expression was not observed, as 1+ when partially positive but did not include more than 25% of epithelium or inflammatory cells, as 2+ if the positive expression was revealed in 26~50%, and as 3+ when more than 50% of epithelium or inflammatory cells reveal positive expression. To identify cell types of VEGF positive cells, we compared H&E and immunohistochemical stains for VEGF in adjacent sections from 3μm thick serial sections.

The expression of Fn was grossly divided into network and non-network patterns. A non-network pattern is composed of focal aggregated or bundle patterns and band patterns. In the network pattern, it was recorded as 0 when there was no Fn expression, as 1+ if there was weak positive reaction in any part of the interstitium, as 2+ when diffuse weak positive reaction was mixed with a strong positive reaction in parts, and as 3+ when showing diffusely strong positive reaction. In the non-network pattern, it was recorded as 1+ if the aggregation or band pattern showed weak positive reaction with unclear boundary, as 2+ if there was even one area showing clear border with strong positive reaction, and as 3+ when many clear-bordered aggregations or band patterns with positive reaction were seen. Toluidine blue staining

was done in adjacent 3μm thick serial sections to assess Fn expression in mast cells.

To compare the VEGF and Fn expression, the total score of each tissue was calculated by summation of each expression score [Fn: the sum of network pattern score and non-network pattern score (scale 0 to +6), VEFG: the sum of scores counted in surface epithelium, glandular epithelium and infiltrating cells (scale 0 to +9)].

5. Statistical Analysis

Data were analyzed by means of SPSS for Windows, version 10.0 (SPSS, Inc, Chicago, III). To evaluate the association between the morphological types of nasal polyposis and accompanying expression of eosinophils, VEGF, and Fn, trends between those ordinal variables, Pearson's Chi-square test and linear-by-linear association were used as appropriate. The variances between the groups of steroid treated and non-treated were also statistically analyzed with Pearson's Chi-square or Fisher's exact test. For analyses of steroid treatment, we adjusted for histologic types using Cochran-Mantel-Haenszel statistics. A P value of less than 0.05 was considered statistically significant.

RESULTS

1. Light microscopic findings

When assorted by topical steroid use, tissue types were significantly different between the two groups (Chi square, P= 0.001 between group I and II, Table 1). The edematous type reached about half in number within the steroid nave group (group I) while no one in the steroid-treated group had this type (group II).

Most of the surface epithelium of NPs was composed of ciliated pseudostratified columnar epithelial cells, but some areas were flat and devoid of cilia. Rarely, denuded or replaced by metaplastic keratinized squamous epithelium was observed. The infiltration of eosinophils or plasma cells was not seen in any NNM tissue. However, eosinophilic infiltration was observed in the half of steroid-nave NP tissues in various degrees from localized migration in lamina propria to heavy infiltration reaching epithelium. The eosinophilic infiltration was more frequent (48.8% vs. 33.3%) and

Table 1. Comparison of histologic types of nasal polyp between the steroid-naïve group (Group I) and the steroid-treated group (Group II)

Type	Group I (n=41)		Group II (n=18)	
	n	%	n	%
Edematous	20	48.8	0	0.0
Glandular/cystic	13	31.7	11	61.1
Fibrous	8	19.5	7	38.9

P=0.001 between groups using the Chi-square test.

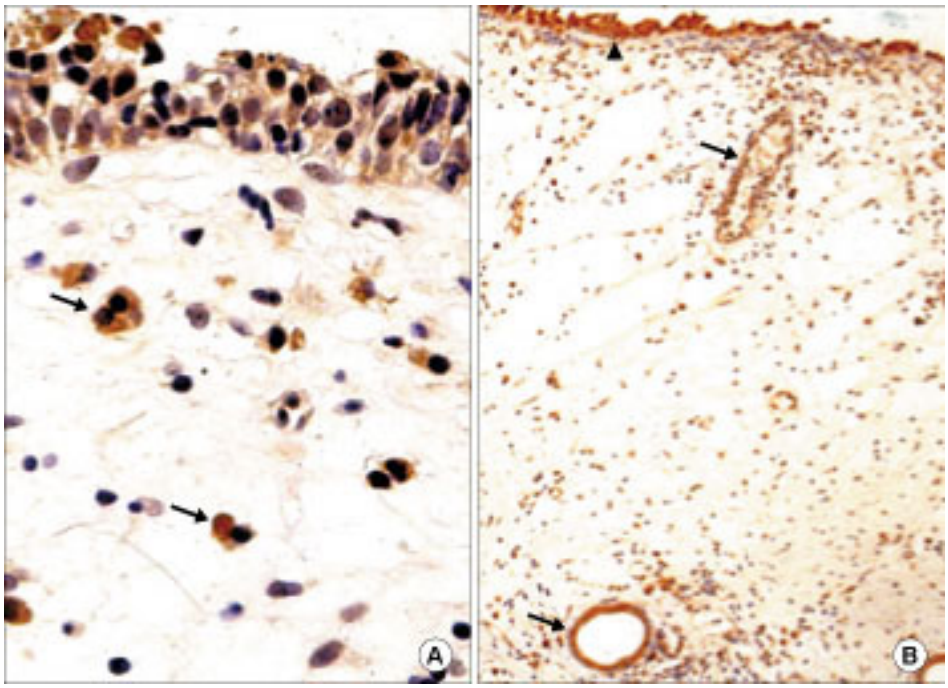

Fig. 1. Immunohistochemical staining for VEGF expression in nasal polyp shows positive reaction in submucosal infiltrates of inflammatory cells (small arrow, A), surface epithelium (arrow head, B), glandular duct epithelium (large arrow, B), and endothelial cells (small arrow, B). (A) ×400, (B) ×50.

more intense (16.7% vs. 0% of 3+ degree eosinophilic infiltration) in steroid-nave group than steroid-treated group. According to histologic types, eosinophilic infiltration was most common in the edematous type (61.9%), and less frequently in the glandular/cystic (37.5%) and fibrous type (40%) but this difference was statistically insignificant. Plasma cells also did not show any statistical difference according to histologic types or topical steroid use.

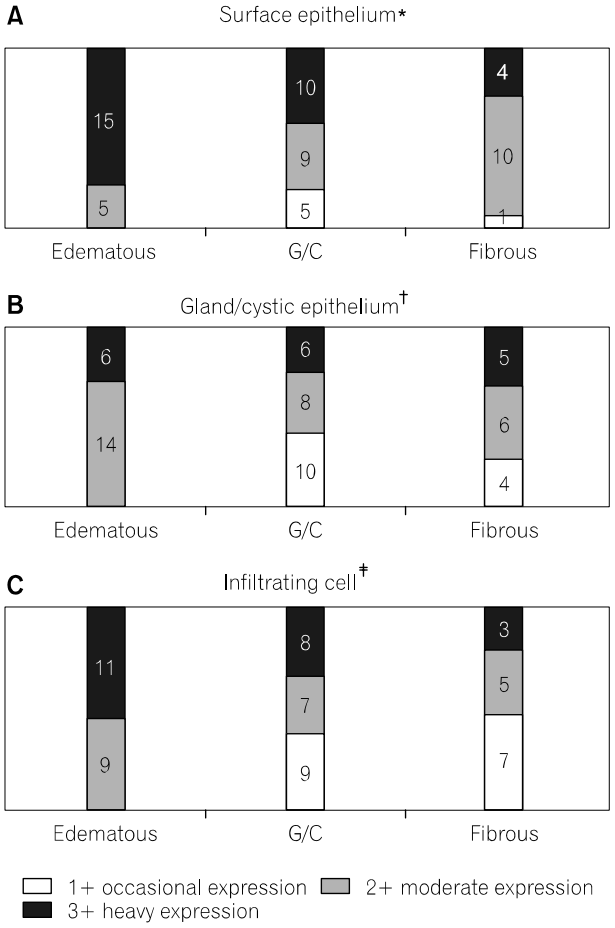

Fig. 2. Different immunoreactivity for VEGF in surface epithelium (A), glandular epithelium (B), and infiltrating cell (C), according to histologic types of nasal polyp. *P=0.01 by Chi-square test; †P=0.020 by Chi-square test; ‡P=0.015 by Chi-square test; G/C = glandular and cystic type.

2. Pattern and degree of VEGF expression

While VEGF was rarely expressed in NNM tissue, all of the NP tissues expressed VEGF. It was expressed mainly in surface epithelium, glandular epithelial cells, endothelial cells, and inflammatory cells, especially plasma cells (Fig. 1). Even though we did not confirm by double staining, the plasma cells were morphologically identifiable in H&E and VEGF stains. The degree of VEGF expression was significantly different according to the histologic types. VEGF expression was most intense in edematous type, followed by the glandular/cystic and fibrous types and this linear association between VEGF expression and histologic types was significant in surface epithelial cells and inflammatory cells (Linear by linear association, P=0.011 and P=0.002 respectively, Fig. 2). The degree of eosinophil infiltration was not correlated with the degree of VEGF expression. According to topical steroid use, VEGF expression was much lower in steroid-treated group compared to steroid-nave group ($P<0.001$, Table 2).

3. Pattern and degree of Fn expression

The expression of Fn was shown as the network pattern, either as local or diffuse form, at the severely edematous lamina propria. In other parts, a focally aggregated or bundle-like pattern of non-edematous area and fibrous lamina propria, and a band-like pattern along the basement membrane were also observed (Fig. 3). Sometimes, it was expressed in a granular pattern in the cytoplasm of inflammatory cells. Serial toluidine blue staining revealed that mast cells were one of the Fn-positive cells. Fn expression was observed in more than half of the NP tissue (the network pattern in 67.8%, non-network pattern in 62.7% in NP tissue), while only one of 9 NNM tissues showed weakly positive expression of Fn. Fn expression was related with histologic types rather than steroid

Table 2. Comparison of VEGF expression in nasal polyp between the steroid-naive group (Group I) and the steroid-treated group (Group II)

Location	Group I (n=41)			Group II (n=18)		
	1+	2+	3+	1+	2+	3+
Surface epithelium	0	12	29	6	12	0
Glandular epithelium	0	24	17	14	4	0
Infiltrating cell	1	18	22	15	3	0

1+ = occasional expression; 2+ = moderate expression; 3+ = heavy expression. $P<0.001$ between two groups using the Chi-square test.

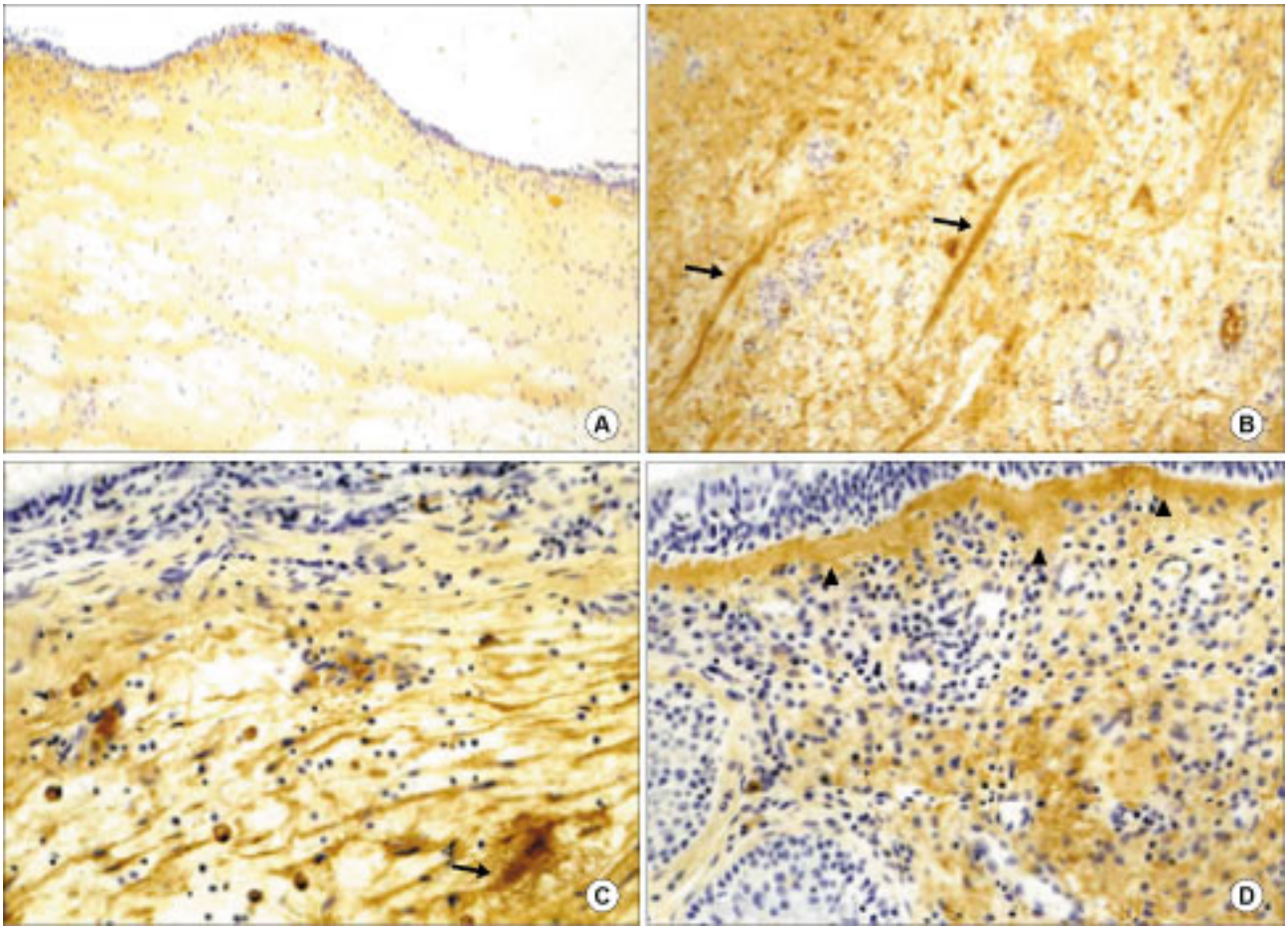

Fig. 3. Immunoreactivity for fibronectin shows diverse patterns: (A) network pattern in severely edematous lamina propria (×100), (B) bundle-like pattern (arrow, ×100), (C) focally aggregated pattern (arrow, ×200), and (D) band-like dense deposition along the basement membrane (arrow head, ×200).

treatment in either pattern. The intensity of Fn expression was higher in the order of edematous, glandular/cystic, and fibrous type (Fig. 4). Edematous type showed most intense Fn expression in both the network pattern and non-network pattern. There was no significant association between Fn expression and eosinophil infiltration. Degree of Fn and VEGF expressions showed considerable linear accordance with each other (Fig. 5). Tissues with more VEGF positive cells had more intense Fn expression and that association was found between VEGF and Fn expression regardless of cell types.

DISCUSSION

Eosinophilic infiltration and inflammatory changes are prominent features of NP tissue. Previously Kakoi and Hiraide[10] reported that the eosinophilic infiltration in NP tissue was more frequently observed in the edematous type (73%) and the glandular/cystic type (52%) compared to the fibrous type (13%). They concluded that edematous and glandular/cystic types were related to an acute stage of tissue reaction and injury while fibrous type was more related to the healing phase. In the present study, eosinophilic infiltration was most common in the edematous type (61.9%) and this finding was in accordance with previous reports. Interestingly, the fibrous type, which has been regarded as a healing stage, also revealed eosinophilic infiltration in a large number (40%).

VEGF is known to increase microvascular permeability as well as enhance angiogenesis.[12,13] Vento et al. reported that VEGF expression was rather low in the epithelium of NP tissue compared with control.[11] However, in accordance with our study, other studies have reported an increased expression of VEGF in NP tissue.[6,7] In this study, VEGF expression was increased in the

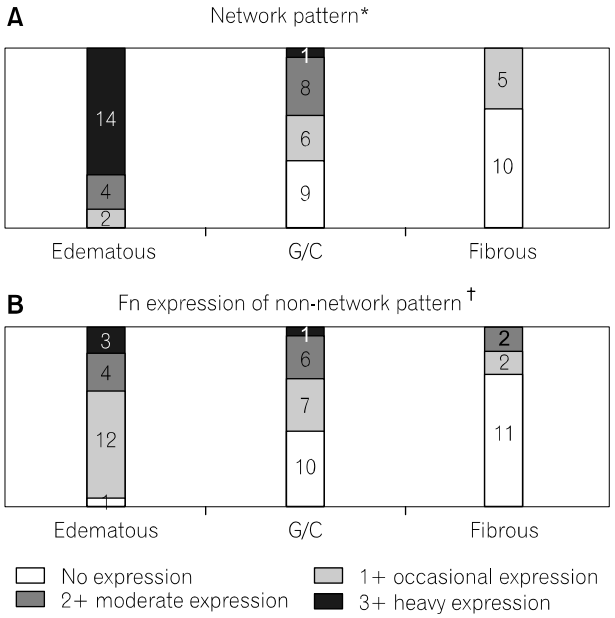

Fig. 4. Different fibronectin expression of network pattern (A) and non-network pattern (B), according to histologic types of nasal polyp. *$P<0.001$ by Chi-square test; †$P=0.02$ by Chi-square test; G/C = glandular and cystic type.

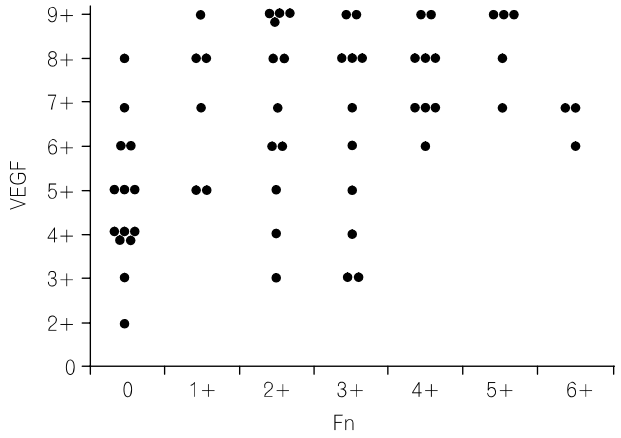

Fig. 5. Association between Fn expression (the sum of network pattern score and non-network pattern score; scale 0 to 6+) and VEGF expression (the sum of scores counted in surface epithelium, glandular epithelium and infiltrating cells; scale 0 to 9+). Linear by linear association, $P=0.001$.

edematous type and to a lesser extent in the glandular/cystic and fibrous type. This reflects that VEGF may play a major role in edematous change of NP. Ito et al.[14] have previously shown that the production of VEGF in plasma cells by *in situ* hybridization method. In our study, many plasma cells were positive for VEGF staining, suggesting that plasma cells are one of the key inflammatory cells in the process of NP formation by secretion of VEGF.

Fn has been known to participate in adhesion, migration, differentiation, proliferation, and the healing process of injured respiratory epithelium.[15,16] It can activate eosinophils and other inflammatory cells through binding of the CS-1 moiety and intergrin α4β1 as well as prolong the survival of eosinophils.[17-19] In this study, Fn was expressed mainly in the lamina propria, but also positively stained in the infiltrated inflammatory cells, including mast cells. Although we could not find the association between the Fn expression and eosinophil infiltration as Nakagawa et al. previously reported,[8] the expression of Fn was more marked and frequently observed in the edematous type of polyps. This finding is in accordance with the previous report that Fn expression was increased during the active phase of tissue inflammation, especially tissue edema.[8] Recently, VEGF was known to bind to Fn and also induces its secretion in human airway smooth muscle cells.[20,21] In our study, both VEGF and Fn were highly expressed in NP tissue in order of edematous, glandular/cystic, and fibrous types. Intensity patterns of VEGF and Fn expression were quite similar in each histologic types, and both expressions showed significant correlation with each other. These findings suggest that both of them have a close connection with each other and their production is important in determining the histologic type and tissue remodeling of NP.

At present, corticosteroids are regarded as the most effective medical therapy for nasal polyposis. In addition to the well-known effects of steroids, such as, suppression of eosinophilic infiltration and chemotaxis, down-regulatation of eosinophilic adhesion molecules in vascular endothelial cells, and shortening eosinophil survival,[22-25] corticosteroids were proved, in *in vitro* experiment, to suppress the VEGF expression by blocking the effects of IL-1β in the induction of *VEGF* mRNA.[26] In the present study, the frequency of edematous type polyps and the expression of VEGF were significantly reduced in the steroid-treated group. These findings suggest that one effective mechanism of topical steroids in NP may be to suppress the production of VEGF and, as a result, improve an acute inflammatory phase to a healing phase.

This study had a few limitations: specifically, retrospective evaluation, relatively small number of patients, semi-quantitative ordinal scaling, and heterogeneity of treatment period. However, our study clearly shows that the production of VEGF and Fn is significantly increased in NP tissues and closely related with their histologic types, particularly at inflammatory stage, and suppression of VEGF production may be one mechanism of therapeutic

effects of topical steroid therapy.

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