Vascular Endothelial Growth Factor in Henoch-Schonlein Purpura

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ABSTRACT. ***Objective.*** To investigate the possible role of vascular endothelial growth factor (VEGF) in the pathogenesis of Henoch-Schonlein purpura (HSP).

Methods. Plasma VEGF levels were determined in 22 children by ELISA. Ten age matched healthy children served as controls. VEGF expression was evaluated by immunohistochemistry within the cutaneous vasculitic lesion as well as the nonaffected skin and in the skin specimens during the resolution of the disease.

Results. Plasma VEGF levels in pg/ml (mean ± SE) were significantly higher during the acute phase (407.8 ± 64.92) when compared with the levels seen during the resolution phase (202.17 ± 26.6; $p < 0.002$) and in healthy controls (135 ± 22.8; $p < 0.001$). Analysis showed that there was a correlation with erythrocyte sedimentation rate, C-reactive protein, white blood cell and platelet count. In all skin specimens, the intensity of the staining of VEGF in the epidermis, dermis, and vascular endothelial bed were evaluated and scored from (+) to (++++). VEGF expression in the epidermis and the vascular bed was more intense in resolving lesions compared with acute vasculitic lesions ($p < 0.05$).

Conclusion. Our results suggest that as a potent permeability, chemotactic, and migratory factor, VEGF may play a crucial role in the morphological and functional changes of the vascular bed and inflammatory reaction in HSP. (J Rheumatol 2001;28:2269–73)

Key Indexing Terms:
VASCULAR ENDOTHELIAL GROWTH FACTOR HENOCH-SCHONLEIN PURPURA VASCULITIS

Vascular endothelial growth factor (VEGF) is a potent mitogen for micro and macrovascular endothelial cells as well as vascular smooth muscle cells and pericytes, and has a crucial function in the regulation of physiological and pathological growth of blood vessels. Although most organs become vascularized during embryogenesis, new blood vessels during adulthood are formed in the reproductive organs and during pathological conditions of inflammation, tumorigenesis, tissue ischemia, or diabetes. VEGF is also closely linked with the formation and maintenance of normal vessel integrity via vascular endothelin cadherin. Besides its crucial role in angiogenesis and in maintaining vascular integrity, VEGF stimulates functional changes in endothelial cells that include increased vascular permeability to fluids and plasma proteins, interstitial collagenase production, von Willebrand factor release and enhanced procoagulant activity[1-7].

Henoch-Schonlein purpura (HSP) is the most common type of vasculitis in childhood. It is a leukocytoclastic vasculitis, which is the result of a complex series of inflammatory and immunologic processes. Since vascular endothelial injury is the key factor in HSP and VEGF stimulates functional changes in endothelial cells, it is very likely that VEGF may play a role in the disease.

We sought an association between VEGF and HSP by analyzing the plasma levels of VEGF and expression of VEGF by immunohistochemistry in HSP within the active and resolving cutaneous lesions as well as the nonaffected skin of children with HSP.

MATERIALS AND METHODS

Patients. Thirty-four patients with HSP, confirmed by a skin biopsy that revealed leukocytoclastic vasculitis, were evaluated. The control group consisted of 10 age matched children for the determination of plasma VEGF levels. For the immunohistochemistry control group, 10 histologicaly normal skin sections from patients undergoing plastic surgery were used.

Laboratory evaluation. The patients were receiving no medication at the time of serum sampling and skin biopsy in the acute phase of the disease. Serum samples were analyzed both during the acute phase of the disease and after remission for erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), C3, C4, p-ANCA, and VEGF levels. Plasma VEGF levels

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were measured by ELISA, using a commercial kit (R&D Systems, Oxon, UK) in accordance with the manufacturer's instructions. Since the individual comparisons between serum and plasma VEGF levels confirmed that higher levels determined in serum are due to the secretion of VEGF by platelets during the clotting process, we used citrate-collected plasma samples for measuring VEGF levels[8]. Results (mean ± SE) are expressed as pg/ml. Elevated plasma VEGF levels were defined as being higher than the value of the mean + 2 SD (135 + 2 × 64.7) in the normal subjects. This resulted in a cutoff value for VEGF of 265 pg/ml.

Skin biopsies from affected and nonaffected skin were obtained from all 34 patients, but in 24 patients, 6–8 weeks after recovery. Light and immunofluorescence microscopy and immunohistochemical staining were carried out for assessing the skin biopsy specimens.

Immunofluorescence. Five micrometer frozen sections were collected on poly-L-lysine coated slides and used for immunofluorescence with the FITC conjugated rabbit anti-human IgA (Dako, Wycombe, UK). Sections were fixed in cold acetone for 10 min and incubated with FITC conjugated IgA (1/10 dilution) for 1 h at room temperature. Sections were washed, dried, and examined under fluorescence.

Immunohistochemistry. Formalin fixed and paraffin embedded 5 μm thick sections were collected on poly-L-lysine coated slides and used for immunohistochemistry with rabbit-anti-VEGF (Biogenex, San Roman, CA, USA). Immunostaining was achieved using an avidin-biotin complex peroxidase method[9]. Sections were dewaxed with xylene and rehydrated with graded ethanol solutions. Endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide solution in 80% methanol for 10 min. Antigen retrieval was carried out by heating (5 min each) in a microwave oven in a 10 mmol/l citrate buffer at pH 6.0. Sections were incubated with the primary anti-VEGF antibody (Biogenex) for 2 h at room temperature with an optimal dilution of primary antibody (1:10). The avidin-biotin complex peroxidase detection system (Biogenex) was used as the second and third steps. The peroxidase reaction was visualized using a 3,3-diaminobenzidine tetrahydrocloride H_20_2 (Biogenex) and sections were counterstained with hematoxylin. Negative controls were carried out by replacing the primary antibody with phosphate buffered saline. The specificity of the staining was assessed by incubating the diluted anti-VEGF antibody with human recombinant VEGF (R&D Systems) overnight at 4°C prior to use in the staining. Epidermis, dermis, and vessel wall were all evaluated. The staining intensity was graded from weak (+) to strong (++++).

Statistical analysis. Paired t Student and McNemar tests were used for comparing the groups. Spearman analyses were used for correlation studies. Data were analyzed using SPSS+PC and results were given as mean ± standard error. A p value ≤ 0.05 was considered significant.

RESULTS

We studied 34 patients, 19 boys and 15 girls, with a mean age of 9.2 ± 0.5 years. All patients presented with the characteristic palpable purpuric skin lesions of HSP. Twenty-two (61%) and 27 (75%) had gastrointestinal system involvement and arthralgia/arthritis, respectively (Table 1). Ten had renal involvement, 8 presented with microscopic hematuria and/or trace to mild proteinuria, one had gross hematuria, and one had rapidly progressive glomerulonephritis

Serum ESR, CRP levels, WBC and platelet count were significantly higher during the acute phase when compared with the resolution phase (Table 2). Serum IgA level was increased in 22 of 34 patients. Serum complement C3 and C4 levels were normal. P-ANCA was found to be negative in 16 and positive in 4 of 20 patients tested. HBsAg was negative in all but one.

Table 1. Patient demographics.

Total number of patients	34
Mean age, yrs (range)	9.2 ± 0.5 (4–15)
M/F	19/15
Patients with	
Cutaneous vasculitis	34
Arthralgia/arthritis	27
Renal involvement	10
Gastrointestinal involvement	22
Determination of plasma VEGF level	22
Skin biopsies from affected skin	34/34
Skin biopsies from nonaffected skin	34/34
Skin biopsies after recovery	24/34

Circulating VEGF levels were measured in plasma samples to avoid the secretion of VEGF by platelets during the clotting process in serum. Plasma VEGF levels were significantly higher (407.8 ± 65) in the acute phase when compared to those in the resolution phase (202.2 ± 26.6) and controls (135 ± 22.8) (p = 0.002 and 0.001, respectively) (Table 2). According to the cutoff level of 265 pg/ml, elevated levels were observed in 20 of 22 patients (91%). VEGF levels of patients in the acute and resolution phases have been plotted in Figure 1. Plasma VEGF levels failed to show any difference between the patients with and without renal involvement as well as gastrointestinal and joint involvement.

There was a significant correlation between plasma VEGF levels and ESR (r = 0.55, p = 0.007), WBC (r = 0.41, p = 0.05), platelet count (r = 0.40, p = 0.05), and CRP (r = 0.40, p = 0.05) in the acute phase of the disease.

Light microscopy of skin biopsies of the purpuric lesion during the acute phase showed a leukocytoclastic vasculitis and IgA immune deposits were detected in dermal vessels (Figures 2A and 2B). Skin samples obtained from unaffected skin during the acute phase and after recovery were normal.

Immunohistochemical staining revealed cytoplasmic staining localized to the endothelial cells, also around the

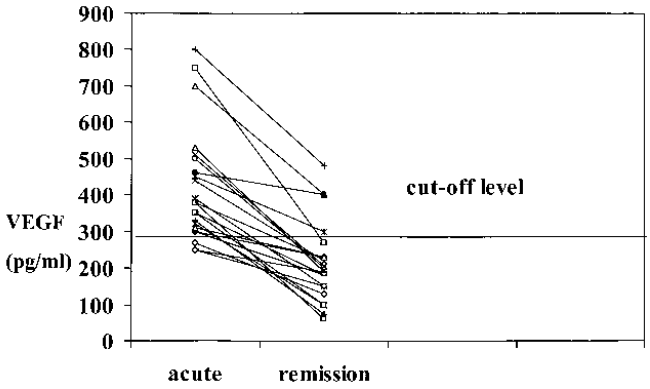
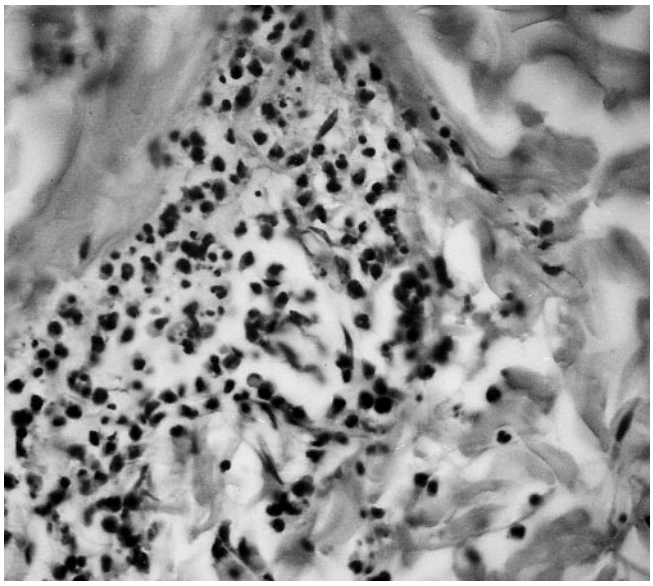


Figure 1. Plasma VEGF levels of the patients in acute and resolution phase. Twenty-two of 24 patients had VEGF levels above cut-off level in acute phase.

Table 2. VEGF levels and acute phase reactants in acute and resolution phases.

	VEGF, pg/ml, Mean ± SE	WBC, mm^3	Platelet, mm^3	ESR, mm/h	CRP mg/dl
Acute	407.8 ± 65	13664 ± 940	465800 ± 5110	41.2 ± 3.6	1.6 ± 0.2
Resolution	202.2 ± 26.6	8277 ± 342	441866 ± 4123	20.6 ± 2.2	0.4 ± 0.7
p	0.002	0.001	0.003	0.001	0.001

VEGF: vascular endothelial growth factor; WBC: white blood cell count; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein.



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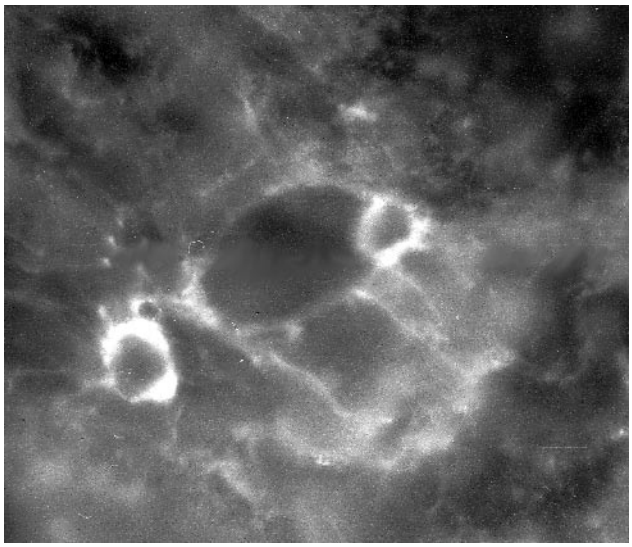

B

Figure 2. A. Infiltration of mostly neutrophils and nuclear dust is present both around and in the walls of small blood vessels (H&E, ×115). B. IgA immune deposits in dermal vessels (immunofluorescence, ×460).

vessel wall and basal and suprabasal levels of epidermis. VEGF staining intensity is shown in Table 3 in various groups. While weak (+) staining of the epidermis was observed in the vasculitic lesion, weak to intermediate (+/++) staining was observed in basal and suprabasal layers of the epidermis in nonaffected skin sections of the acute phase (Figures 3 and 4). The expression of VEGF was intermediate to strong (++/+++) in both basal and suprabasal layers of the epidermis, in the resolution phase (Figure 5). Similar VEGF staining intensity was observed in the dermis and the vessel wall in acute and resolution phases (Figures 3 to 5). The vascular VEGF staining was significantly more intense in the resolution phase compared to the acute phase ($p < 0.05$) (Figures 3 and 5), while there was no difference between the resolution phase and the nonaffected skin of the acute phase (Figures 4 and 5). The most intense staining of the vascular wall (++++) was observed in histologicaly normal healthy skin sections.

DISCUSSION

HSP, a small vessel vasculitis, is the consequence of inflammatory and immune reactions mediated by a complex series of endothelial/leukocyte interactions, vascular dilatation and leakage, deposition of immune complexes, and perivascular C3 deposition. The activation of mast cells, eosinophils, and neutrophils also contributes through the release of cytokines to endothelial swelling, necrosis, and fibrin deposition. Endothelial adhesion molecules, increased expression of interleukin 1 (IL-1), tumor necrosis factor (TNF-α), and impaired fibrinolysis are considered to play a very important role in the induction of vascular endothelial cells and immunoinflammatory reactions in HSP[10-13].

Table 3. Intensity of VEGF staining in groups.

	Epidermis Basal/Suprabasal	Dermis and Vessel Wall
Acute phase	+/+	+/+
Resolution	++/+++	++/+++
Acute phase nonaffected lesion	+/++	+/++
Normal	+/++	+++/++++

Staining intensity was scored + weak, ++, +++ moderate, and ++++ strong.

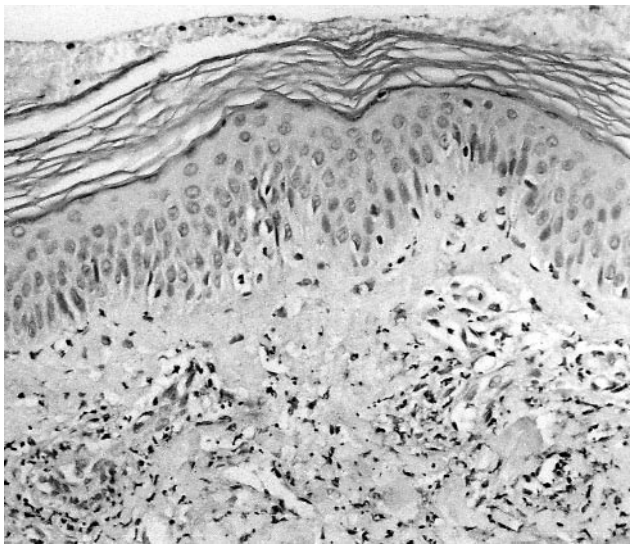

Figure 3. There is weak positivity in endothelial cells and epidermis in acute phase of HSP (immunoperoxidase, ×460).

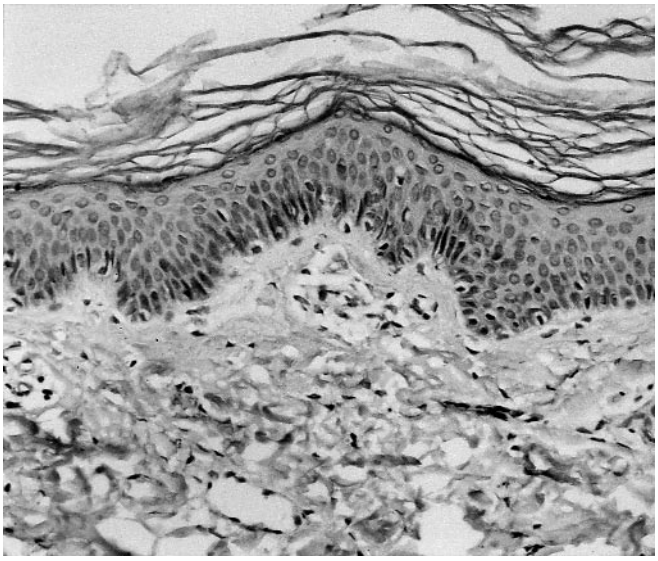

Figure 4. Endothelial cells and epidermis show mild/moderate positivity in nonaffected skin of acute phase (immunoperoxidase, ×460).

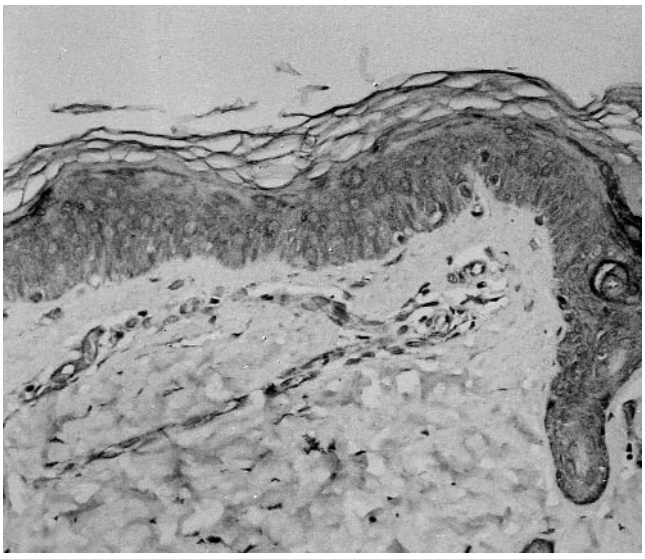

Figure 5. Endothelial cells and epidermis show moderate and strong positivity in resolution phases (immunoperoxidase, ×460).

On the other hand, VEGF is known as the most potent vascular permeability factor, which stimulates functional changes in endothelial cells. It is involved in the transendothelial migration of monocytes and is chemotactic for mast cells[6,7,14]. VEGF upregulates the expression and activity of plasminogen activators, urokinase-type plasminogen activator (uPA), and tissue plasminogen activator (tPA) and their inhibitor PAI-1. These serine proteases convert plasminogen to plasmin and are involved in tissue remodeling, cell invasion, and thrombolysis[15]. Furthermore, proinflammatory cytokines such as IL-1, IL-6, transforming growth factor (TGF-α), and platelet derived growth factor (PDGF), reactive oxygen species (ROS), angiotensin II, and hypoxia stimulate VEGF expression and/or production[7]. Given these findings, VEGF may have a role in the pathogenesis of inflammation and vasculitis. As an example, patients with rheumatoid arthritis have high VEGF levels in their sera and synovial tissues[16].

We found a significant increase in the plasma levels of VEGF in the acute phase of HSP. VEGF levels were correlated with the ESR, CRP, WBC and platelet count in the acute phase of the disease. These elevated VEGF levels decreased to near normal in remission, which correlated with clinical improvement. Patients with various kinds of vasculitis, such as Wegener's granulomatosis and with Kawasaki disease, are also reported to have markedly elevated VEGF levels in the acute phase of the disease[17,18]. In our study, while plasma VEGF levels were elevated in the acute phase, the expression of VEGF in the vascular wall and epidermis of affected skin was less intense than in resolving lesions (Figures 3 and 5).

These results show that this factor may contribute to the disease process in several ways and in different phases of the disease. As a permeability factor, high VEGF may initially induce erythema and endothelial cell swelling. As a consequence of increased vascular permeability, fibrin and immune complexes may be deposited and altered perivascular stroma and monocyte migration may be promoted. However, in our study, VEGF expression was not increased in the vascular wall during the acute phase, perhaps since some other sources participate in the production of VEGF, including activated platelets, neutrophils, and epidermis[19]. VEGF was expressed more in the vascular wall of the resolving lesion, which may be in agreement with a recent study[7]. Since in that study VEGF expression was found in sites where no angiogenesis occurs and high affinity VEGF

binding was present in quiescent endothelial cells, Ferrara, *et al* hypothesized that VEGF in the vascular wall is not limited to the induction of growth *per se*, but is involved in preventing apoptosis of endothelial cells, in regression of blood vessels, and in regulating vessel function under physiologic conditions[7].

Our results showed that plasma VEGF levels are raised in the acute phase of HSP compared to the resolution phase and may be used as a marker of disease activity. Further, altered intensity of vascular VEGF expression in the acute phase compared with the resolution phase suggests that VEGF may play a crucial role in morphological and functional changes of endothelial cells and inflammatory reactions in HSP.

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