

WE investigated the serum concentration of vascular endothelial growth factor (VEGF) and its two soluble receptors, sVEGFR-1 and sVEGFR-2, in a group of 60 patients with systemic lupus erythematosus (SLE), and 20 healthy controls, using an enzyme-linked immunosorbent assay. We examined a possible association between serum levels of these proteins and certain clinical and laboratory parameters as well as SLE activity. VEGF, sVEGFR-1 and sVEGFR-2 were detectable in all patients with SLE and in all normal individuals. The VEGF level was higher in active SLE (mean, 300.8 pg/ml) than in inactive SLE (mean, 165.9 pg/ml) ($p < 0.05$) or in the control group (mean, 124.7 pg/ml) ($p < 0.04$). The highest sVEGFR-1 concentrations were also detected in active SLE patients (mean, 42.2 pg/ml) and the lowest in inactive disease (mean, 32.0 pg/ml) ($p < 0.01$). In contrast, the levels of sVEGFR-2 were lower in SLE (mean, 12557.6 pg/ml) than in the control group (mean, 15025.3 pg/ml) ($p < 0.05$). We found a positive correlation between sVEGFR-1 concentration and the SLE activity score $\rho = 0.375$ ($p < 0.004$) and a negative, but statistically insignificant correlation between sVEGFR-2 and SLE activity ($\rho = -0.190$, $p > 0.05$). Treatment with steroids and cytotoxic agents did not influence VEGF or its soluble receptors levels. In conclusion, in SLE patients the levels of VEGF and sVEGFR-1 are higher in patients with active SLE than in inactive disease or healthy persons. In contrast, the level of sVEGFR-2 is lower in active SLE than in inactive disease. The imbalance between VEGF and its soluble receptors may be important in SLE pathogenesis.

Key words: Angiogenesis, VEGF, Soluble VEGF receptors, FLK-1, *fms*-like tyrosine kinase 1, Systemic lupus erythematosus, Disease activity

Vascular endothelial growth factor and its soluble receptors VEGFR-1 and VEGFR-2 in the serum of patients with systemic lupus erythematosus

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Introduction

Vascular endothelial growth factor (VEGF) is a key regulator of vasculogenesis and angiogenesis.^{1,2} VEGF is produced by endothelial cells, macrophages, fibroblasts and smooth muscle cells.¹ It is a chimeric glycoprotein with a molecular weight of 34–45 kDa, consisting of two subunits.^{3,4} Five isoforms of human VEGF have been described to date, each generated by alternative splicing of a single mRNA and resulting in proteins of varying amino acid length, (VEGF).^{121,145,165,189,206} This angiogenic cytokine binds to receptors on endothelial cells and acts as direct inducer of angiogenesis both *in vivo* and *in vitro*.⁵ The two best characterised VEGF receptors are termed VEGF receptor 1 (VEGFR-1) and VEGF receptor 2 (VEGFR-2). VEGFR-1 (*fms*-like tyrosine kinase 1) and VEGFR-2 (kinase domain receptor/FLK-1) are specific tyrosine kinase receptors that together with platelet-derived growth factor re-

ceptors form the subtype III of tyrosine kinase receptors.^{2,6–8} The gene for VEGFR-1 is almost exclusively expressed on endothelial cells but is also found on monocytes.⁹ VEGFR-2 is not found on monocytes. Both receptors share common features such as seven immunoglobulin-like extracellular domains, a single transmembrane region and a consensus tyrosine kinase sequence interrupted by a kinase insert domain, and they are highly glycosylated.^{10,11} However, although VEGFR-1 binds to VEGF with substantially higher affinity, most of the biological effects of VEGF seem to be mediated via VEGFR-2.¹²

Recently, a naturally occurring soluble form of VEGFR-1 (sVEGFR-1) has been identified, but naturally occurring secreted forms of sVEGFR-2 have not been reported to date.^{11,13–15} The physiological role of sVEGFR-1 and sVEGFR-2 is still undetermined. It has been shown that sVEGFR-1 retains its high-affinity binding to VEGF and it is likely to be a

negative regulator of VEGF availability, or that it may prolong the different VEGF activities.^{13,16–18}

Angiogenic cytokines and angiogenesis inhibitors play an important role in the pathogenesis of several diseases including connective tissue diseases.^{19–22} In our previous studies we have shown that in SLE patients the serum level of some angiogenic cytokines is higher than in healthy controls, but the level of endostatin, the endogenous angiogenesis inhibitor, is similar in SLE and in the control group.^{21,22} Moreover, selected pro-angiogenic cytokines correlated with SLE activity.

In the present study we measured the serum concentrations of VEGF and its soluble receptors VEGFR-1 and VEGFR-2 in patients with SLE using an enzyme-linked immunosorbent assay. The serum levels of these proteins were also correlated with disease activity and some clinical and laboratory parameters. To the best of our knowledge, the serum levels of sVEGFR-1 and sVEGFR-2 in patients with SLE have not been investigated to date.

Patients and methods

The study group consisted of 60 patients with SLE (55 females and five males) and 20 sex-matched and age-matched healthy volunteers. The median age of the SLE patients was 41 years (range, 18–75 years). The diagnosis of SLE was based on the revised criteria of the American Rheumatism Association.²³ The mean duration of the disease was 71 months (range, 3 months–24 years). Twenty-four patients were never treated with steroids or any other immunosuppressive agents. Fourteen patients were treated with prednisone at a dose of 5–20 mg/day during the study and five patients with prednisone and azathioprine or cyclophosphamide. Patients' histories were recorded and physical examination was performed on the day of blood collection. Patients with both active and inactive disease were included in the study. In all patients the activity of the disease was determined according to the systemic lupus activity measure (SLAM) scale.²⁴ Each patient was examined on two separate occasions 2–4 weeks apart. The SLAM system includes 24 clinical manifestations and eight laboratory parameters. The maximum score in this system is 84 points. In our group of patients, the number of points ranged from 9 to 27. In the present study we considered a score of 0–15 points as inactive disease and a score over 15 points as active disease. By this definition active disease was found in 32 patients and 28 patients had inactive disease.

The clinical and laboratory features of SLE patients are presented in Table 1. A control group of 20 healthy volunteers (17 women and three men) aged from 35 to 58 years (median, 45 years) was also

Table 1. Clinical and laboratory characteristics of SLE patients

Symptoms	Number of patients	%
Total	60	100
Age (years) [mean (range)]	41 (18–79)	
Sex (male/female)	5/55	8.3/91.7
Active/inactive	32/28	53.3/46.7
Fever	15	25.0
Arthritis	54	90.0
Skin symptoms	30	50.0
Reticuloendothelial system involvement	32	53.3
Renal disorder (kreatinine > 1.3 mg/dl)	5	8.3
Neurologic symptoms	35	58.3
Antinuclear antibodies	49	81.6
Immunoglobulins deposit at the dermal–epidermal junction	33	55
Anaemia (haemoglobin < 12 g/dl)	30	50.0
Leukopaenia (white blood cells < 3.5 × 10 ⁹ /l)	20	33.3
Thrombocytopaenia (platelets < 150 × 10 ⁹ /l)	14	23.3
C-reactive protein (> 4.7 mg/L)	15	25.0
Raised ESR (> 25 mm/h)	24	41.6
Treatment with steroids during the study	14	23.3
Treatment with steroids and cytotoxic agents during the study	5	8.3

studied. Each person underwent a thorough physical evaluation by one of the authors (E.R.). The patients with SLE and controls showed no clinical signs of infections or neoplastic disease and were not given antibiotics or any other antibacterial or antiviral medication for at least 4 weeks prior to blood donation. This project was performed in accordance with the Helsinki Declaration and was approved by the local Ethics Committee. Informed consent was obtained from all the patients.

Laboratory tests

In the study group the following laboratory parameters were analysed: complete blood cell count, erythrocyte sedimentation rate (ESR), urinalysis, blood urea nitrogen and creatinine levels, fibrinogen, partial thromboplastin time, alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, immunoglobulins (IgG, IgM and IgA) and complement (C₃, C₄) levels and antinuclear antibodies. Immunoglobulin deposits at the dermal–epidermal junction (lupus band test) were also determined.

Serum sampling and detection of VEGF and its soluble receptors

Venous blood (5 ml) was collected in pyrogen-free tubes, allowed to clot at 4°C for 1 h and centrifuged at 2000 g for 10 min. The sera obtained were allocated

into separate vials and stored at -25°C until assayed for VEGF and its soluble receptors. The serum was randomly coded and the testing was carried out without knowledge of the clinical status of the subject or of any related laboratory data. The cytokines serum concentrations were assayed by specific, commercially available, enzyme-linked immunosorbent assay kits (Qantikine; R&D Systems Inc., Minneapolis, Minnesota, USA) using horseradish peroxidase detection in accordance with the manufacturer's instructions. The absorption was read at 492 nm. In each assay the appropriate recombinant human cytokine or receptor was used to generate the standard curve. Standards as well as samples were assayed as duplicates and the inter-assay variations were shown to be within the range given by the manufacturer. The procedure has previously been described in detail.^{22,25} The sensitivity of the assay for VEGF was < 5.0 pg/ml with intra-assay and inter-assay coefficients of variation of 5.1% ($n = 20$) and 6.2% ($n = 40$) at 0.9 ng/ml and 1.0 ng/ml. The mean minimum detectable dose of VEGFR-1 was 5.01 pg/ml and ranged from 1.63–14.4 pg/ml, with intra-assay and inter-assay coefficients of variation of 3.5% ($n = 20$) and 8.1% ($n = 40$) at 1.5 ng/ml. Serum for the sVEGFR-2 concentration measurement was diluted five-fold. The mean value of minimum detectable dose was 4.6 pg/ml (range, 1.0–11.4 pg/ml) with intra-assay and inter-assay coefficients of variation of 2.9% ($n = 20$) and 5.7% ($n = 40$) at 2.9 ng/ml. The concentrations of VEGF and soluble VEGF receptors in the samples were determined by interpolation from the standard curve.

Statistical analysis

For the statistical analysis of the data the range of measured variable is given (minimum–maximum). The mean arithmetic value, median and standard deviation were also calculated. The medians were compared using the Mann–Whitney test and the Kruskal–Wallis test. The correlation between features

was evaluated using the Spearman rank coefficient p . Comparisons and correlations were considered significant when $p < 0.05$.

Results

In the group of 60 SLE patients, 32 were with active and 28 with inactive disease according to the Liang *et al.* scoring system.²⁴ The serum concentrations of VEGF and its soluble receptors sVEGFR-1 and sVEGFR-2 were detectable in all SLE patients and in healthy volunteers. The results are presented in Table 2. The highest VEGF concentration was found in patients with active SLE (mean, 234.2 pg/ml) and the lowest in the healthy control group (124.7 pg/ml) ($p < 0.04$). No difference in VEGF serum concentrations in inactive SLE compared with normal individuals was observed. The serum level of VEGF was significantly higher in patients with active disease as compared with patients with inactive SLE (Table 2).

The highest sVEGFR-1 concentrations were also detected in active SLE patients (mean, 42.4 pg/ml) and the lowest in inactive SLE patients (mean, 32.0 pg/ml) ($p < 0.01$). The level of this soluble receptor in inactive SLE and in healthy individuals were not statistically different. In contrast, the levels of sVEGFR-2 in active and inactive SLE patients were similar (Table 2). However, the concentrations of this receptor in SLE patients were lower (mean, 12.5 ng/ml) than in the control group (mean, 15.0 ng/ml) ($p < 0.05$). We found a positive correlation between sVEGFR-1 concentration and the SLE activity score ($\rho = 0.375$, $p < 0.004$) and a negative but statistically not significant correlation between sVEGFR-2 and SLE activity (Fig. 1).

We analysed the correlation between serum levels of VEGF and sVEGFR-1 ($\rho = 0.166$), VEGF and sVEGFR-2 ($\rho = -0.053$) as well as sVEGFR-1 and sVEGFR-2 ($\rho = 0.043$). However, the differences were not statistically significant ($p < 0.05$) (data not shown).

Table 2. Serum levels of VEGF and its soluble receptors (sVEGFR-1 and sVEGFR-2) in patients with SLE and control group

Cytokine/receptors	All SLE ($n = 60$) (a)	Active SLE ($n = 32$) (b)	Inactive SLE ($n = 28$) (c)	Control group ($n = 20$) (d)	Statistically significant comparison
VEGF (pg/ml)					
$\bar{x} \pm \text{SD}$	234.2 \pm 209.9	300.8 \pm 250.9	165.3 \pm 153.4	124.7 \pm 59.7	(b)–(c)–(d), $p = 0.05$
Median	150.4	202.4	116.1	123.5	(b)–(c), $p < 0.05$
Range	6.4–920.4	8.5–920.4	6.4–602.4	21.1–218.5	(b)–(d), $p < 0.04$
sVEGFR-1 (pg/ml)					
$\bar{x} \pm \text{SD}$	37.5 \pm 14.5	42.4 \pm 16.1	32.0 \pm 10.1	38.3 \pm 12.5	(b)–(c)–(d), $p < 0.03$
Median	36.3	39.5	31.2	38.25	(b)–(c), $p < 0.01$
Range	9.9–88.2	9.9–88.2	13.8–49.7	18.8–61.2	
sVEGFR-2 (ng/ml)					
$\bar{x} \pm \text{SD}$	12.5 \pm 3.6	12.0 \pm 3.6	13.2 \pm 3.6	15.0 \pm 5.0	(a)–(d), $p < 0.05$
Median	12.0	11.7	12.7	14.2	(b)–(d), $p < 0.04$
Range	5.0–23.2	5.0–19.8	7.9–23.2	8.7–26	

$\bar{x} \pm \text{SD}$, Mean \pm standard deviation.

We also investigated the correlation between the level of VEGF and its soluble receptors with selected clinical and laboratory parameters of the patients with SLE. In the group of 60 patients, 14 were treated with steroids and five with cytotoxic agents. However, the levels of VEGF, sVEGFR-1 and sVEGFR-2 were similar in both groups ($p > 0.05$) (data not shown). In contrast, the serum concentration of VEGF was higher in the patients with immunoglobulin deposit at the dermal–epidermal junction (mean, 288.6 pg/ml) than in the patients without this symptom (mean, 162.2 pg/ml) ($p < 0.02$). The level of sVEGFR-2 was also higher in SLE patients with skin immunoglobulin deposits (mean, 13.2 ng/ml) than in the patients without these deposits (mean, 11.7 ng/ml) ($p < 0.04$). In contrast, sVEGFR-1 was similar in both groups (mean, 38.4 pg/ml and 36.2 pg/ml, respectively; $p > 0.05$). The mean concentration of sVEGFR-1 was higher in SLE patients with anaemia (haemoglobin < 12 g/dl) at 43.3 pg/ml than in the patients with haemoglobin concentration > 12.0 g/dl (31.9 pg/ml) ($p < 0.001$). In contrast, the levels of VEGF and sVEGFR-2 were similar in both groups ($p > 0.05$). The VEGF and VEGFR-1 serum levels were higher in the SLE patients with a high C-reactive protein level (CRP) (> 4.7 mg/l) (mean values, 361.0 pg/ml and 44.1 pg/ml, respectively) than among patients with a low CRP (< 4.7 mg/l) (192.2 pg/ml and 35.2 pg/ml, respectively) ($p < 0.02$ and $p < 0.01$, respectively). The level of sVEGFR-2 was similar in both groups ($p > 0.05$). We also analysed the relationship between VEGF, sVEGFR-1 and sVEGFR-2 serum levels in the patients with and without skin symptoms, neurological symptoms, renal disorders (kreatinine > 1.3 mg/dl), fever, arthritis, antinuclear antibodies, raised ESR (> 25 mm/h) and thrombocytopenia (platelets $< 150 \times 10^9/l$). We found no statistically significant differences in the levels of these cytokines or receptors in particular groups of patients.

Discussion

The results of numerous studies indicate that VEGF is one of the main positive regulators of angiogenesis. We have shown previously that in patients with active SLE the serum concentration of this cytokine is higher than in healthy controls or patients with inactive disease.²¹ We have also observed a positive but not statistically significant correlation between the VEGF concentration and the concentration of angiogenesis-negative regulator (i.e. endostatin).²² In these studies we have been the first to evaluate serum concentrations of VEGF soluble receptors (sVEGFR-1 and sVEGFR-2) in SLE patients and the relationship between these factors as well as their associations

with SLE activity and some SLE laboratory and clinical parameters. The role of these receptors in the pathogenesis of autoimmune diseases has not been known until now.

It has been shown that mRNA for a soluble form of VEGFR-1 was generated by the alternative splicing in human umbilical vein endothelial cells and that sVEGFR-1, as a competitive inhibitor, was able to bind all isoforms of VEGF and to inhibit VEGF-induced endothelial cell proliferation.¹³ It has been suggested that sVEGFR-1 is likely to be a negative regulator of VEGF availability by sequestering the ligand and by forming inactive heterodimers with membrane-bound VEGF receptors. In this way sVEGFR-1 acts as a specific endogenous antagonist of its membrane-bound counterparts. VEGFR-2 is also able to bind VEGF but that receptor ligand complex formation, in contrast to sVEGFR-1, is heparin dependent.¹⁶ Based on these findings, the soluble VEGF receptors may be involved in the pathophysiology of SLE and their interaction with VEGF as a potential VEGF inhibitor and that they may influence the clinical course of SLE and the disease activity.

In our study we have revealed that the sVEGFR-1 concentration is the highest in active SLE, whereas levels of this receptor in inactive disease and in healthy individuals are comparable. To our knowledge soluble receptors of VEGF have not been previously determined in autoimmune diseases. However, a higher sVEGFR level has been detected in serum or plasma of patients with some haematopoietic system neoplasms or renal cancer^{26–28} and also in healthy people.²⁹ In up-to-date studies endothelial cells and monocytes have been found to be the primary source of sVEGFR-1; however, the regulation of switching the transcription from the transmembrane to the soluble form is still not fully elucidated.³⁰ Moreover, it has been shown that hypoxia and also pro-angiogenic cytokines, VEGF and basic fibroblast growth factor cause up-regulation of sVEGFR-1 expression in endothelial cells.^{30,31} The serum level of these angiogenic cytokines in SLE patients appears to be higher,²¹ which might indicate that they are at least partially responsible for the increase in sVEGFR-1 serum concentration. Moreover, the sVEGFR-1 and VEGF complex can prolong the VEGF half-life through prevention of this cytokine proteolysis, lengthening in this way its activity.¹⁷ In our study, however, we have not observed a positive correlation between VEGF and sVEGFR-1 concentrations.

Contrary to sVEGFR-1, the sVEGFR-2 serum concentration in SLE patients was considerably lower than in healthy controls and did not correlate with the disease activity. These differences are difficult to explain. It is, however, known that monocytes/macrophages express a significant level of VEGFR-1

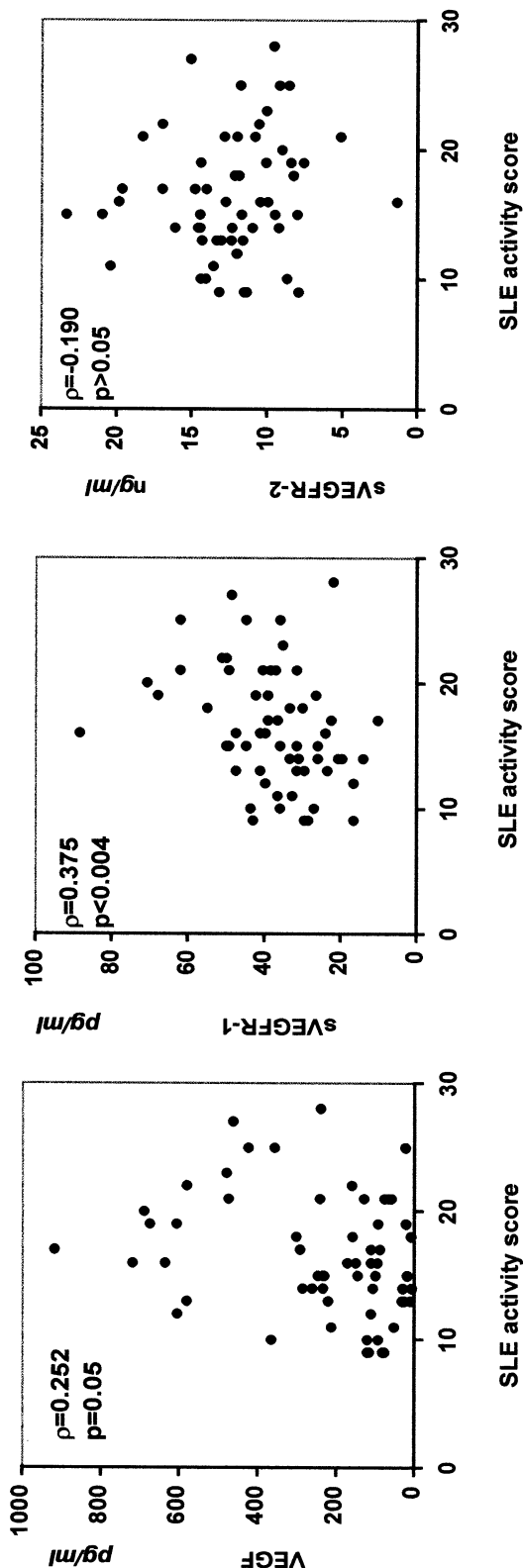


FIG. 1. Correlation between serum levels of VEGF and its soluble receptors and SLE activity.

mRNA but very little, if any, VEGFR-2 mRNA.³² The biological activity of sVEGFR-2 is not completely understood. It has been shown to inhibit corneal neovascularisation induced by conditional media from a rat mammary carcinoma cell line.³³ Furthermore, sVEGFR-2 can increase the number of capillaries displaying synchronous apoptosis in papillary membrane explant assay.³⁴ Yet, the role of this receptor in SLE and other autoimmune diseases pathogenesis *in vivo* requires further investigations.

Differences in biological activity of both receptors should be emphasised. VEGFR-1 has a more than 10-fold higher affinity to VEGF-A, even in a soluble form, but has about a 10-fold lower tyrosine kinase activity than VEGFR-2.³⁵ In addition to sequestering the ligand, sVEGFR-1 can form heterodimers with transmembrane VEGFR-2, prevent its autophosphorylation and thus abolish signalling in a dominant-negative fashion.¹³ In this context the lack of balance between sVEGFR-1 and sVEGFR-2 in serum of SLE patients is likely to have a pathogenetic impact and needs further studies.

In conclusion, in SLE patients the levels of VEGF and sVEGFR-1 are higher in patients with active SLE than in inactive disease or healthy persons. In contrast, sVEGFR-2 is lower in active SLE than in inactive disease. The imbalance between VEGF and its soluble receptors may be important in SLE pathogenesis.

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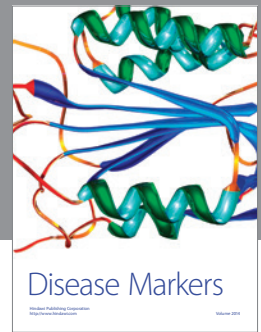
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