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Serum Levels of Vascular Endothelial Growth Factor and Hemoglobin Dielectric Properties in Patients with Systemic Lupus Erythematosus

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The study aimed to assess serum levels of vascular endothelial growth factor (VEGF) in patients with systemic lupus erythematosus (SLE) and to elucidate its correlation with clinical features, laboratory parameters and the disease activity in addition to the effect of SLE activity on hemoglobin dielectric properties. We studied 30 female patients with SLE and 10 healthy female controls. Disease activity was evaluated by SLE disease activity index (SLEDAI) score. Laboratory investigations included complete blood count, erythrocyte sedimentation rate (ESR), urine analysis, 24 h total urinary protein, serum creatinine, ANA, anti-DNA, complement component C_3, lupus anticoagulant and VEGF as well as dielectric relative permittivity (ε') and conductivity (σ) of hemoglobin (Hb) were examined in the range of frequency from 20 to 100 kHz. Serum levels of VEGF were significantly increased in SLE patients (501±120.5 pg mL^{-1}) when compared with controls (65±22.3 pg mL^{-1}) ($p<0.001$). VEGF serum levels were significantly increased in patients having renal involvement than those without ($p<0.001$). SLEDAI score was positively correlated to VEGF serum level ($r = 0.81$, $p<0.0001$). The dielectric results indicated that the studied hemoglobin (Hb) has a dielectric dispersion in the frequency range used. The increase in the electrical conductivity and relaxation time for SLE patients (non-renal) and SLE patients (renal) indicates pronounced changes in the molecular structure of Hb of these patients. The data support the conclusion that VEGF may be relevant to SLE pathogenesis. Its concentration seems to be a marker of SLE activity. SLE may alter dielectric properties of Hb and that probably affected cell to cell communication. VEGF in combination with dielectric dispersion of Hb could help in SLE monitoring and planning of treatment.

Key words: Systemic lupus erythematosus, vascular endothelial growth factor, dielectric, hemoglobin

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized clinically by protean manifestations, most commonly including arthralgia, arthritis, rash, alopecia, oral ulcers, serositis, leukopenia, central nervous system and renal involvement (Lahita, 2004). The disease primarily affects women between the third and fourth decades of life. Even though the etiology of SLE is unknown, many predisposing factors playing an important role have been found, including genetic, environmental, infectious and hormonal factors (McAlindon, 2000). It is an important prototype of systemic autoimmunity and it causes significant disability and premature death in those patients who suffer from it (Lahita, 2004).

The spectrum of disease manifestations among patients with SLE is broad, ranging from subtle symptoms to life-threatening multiorgan failure. Because of its heterogeneous presentation and unpredictable course, clinical management of SLE remains one of the greatest challenges to physicians. The lack of reliable, specific biomarkers for SLE not only hampers precise assessment of disease activity and prompt identification of patients at risk for flares and organ damage, but also prohibits accurate evaluation of responses to treatment (Schiffenbauer *et al*., 2004).

Vascular endothelial growth factor (VEGF) is a key regulator of vasculogenesis and angiogenesis (Plate and Warnke, 1997; Josko *et al*., 2000). VEGF is produced by endothelial cells, macrophages, fibroblasts and smooth muscle cells. It is a chimeric glycoprotein with a molecular weight of 34-45 kDa, consisting of two subunits (Yancopoulos *et al*., 2000; Houck *et al*., 1991). 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. This angiogenic cytokine binds to receptors on endothelial cells and acts as direct inducer of angiogenesis both *in vivo* and *in vitro* (Risau, 1997).

The angiogenic process plays a major role in the development of vascular supply under different pathological conditions such as malignancies and non-neoplastic diseases (Folkman, 1995; Dvorak *et al*., 1995). A family of pro- and anti-angiogenic factors tightly regulates angiogenesis. A large number of cytokines have been shown to stimulate angiogenesis under experimental conditions, including vascular endothelial growth factor (Josko *et al*., 2000).

Several angiogenic growth factors are detectable in human serum or plasma especially in pathological conditions, including neoplastic and collagen diseases. Some of the angiogenic cytokines may have prognostic value. The VEGF serum level was found to be elevated in cancer patients (Fuhrmann-Benzakein *et al*., 2000; Kadono *et al*., 1996; Sezer *et al*., 2001).

VEGF has also been correlated to autoimmune diseases (Matsumoto and Claesson-Welsh, 2001), including systemic lupus erythematosus, where elevated VEGF plasma levels have been associated with disease activity and positively correlated the presence of lupus nephritis. These observations have suggested a possible role for VEGF in systemic lupus erythematosus pathogenesis (Navarro *et al*., 2002). Dielectric properties of various biological materials have been previously investigated to get attractive information about their structural change under any internal or external effect (Polk and Postow, 1996; Ghannam *et al*., 2002; Ibrahim *et al*., 2008).

The objective of this study was to investigate serum levels of VEGF in patients having SLE and the effect of SLE activity on the dielectric properties of Hb. Also we aimed to investigate association of VEGF with SLE activity as well as the clinical and laboratory characteristics of SLE patients particularly nephritis.

PATIENTS AND METHODS

This study was conducted on 30 female patients with SLE and 10 healthy female volunteers of matched ages as control. The SLE patients and matched healthy control were chosen from patients attended the Rheumatology and Rehabilitation Department, Cairo University in 2007. All of them were diagnosed according to the 1982 American Rheumatism Association Revised Criteria for diagnosis of SLE (Tan *et al*., 1982). The median age of the SLE patients was 25 years (range 14-45 years) while the median age of the control group was 32 years (range 20-50 years). Patients' histories were recorded before enrollment in the study. Disease activity was determined in SLE patients by SLE disease activity index (SLEDAI) score. This index takes into consideration 24 variables representing nine organ systems. Each variable is rated as (present or absent) over 10 days before and including the day of the evaluation.

The maximal theoretical score is 105, but in practice few patients have scores greater than 45 (Bombardier *et al*., 1992). According to the clinical and laboratory manifestations of renal disease, patients were categorized into two groups: renal and non-renal SLE groups. Non-renal SLE group included eleven patients (37%) who had no clinical symptoms of renal disease. All had negative protein dipstick tests, protein in urine <0.2 g 24 h^{-1} and no evidence of microscopic or

macroscopic hematuria, pyuria or urinary casts and normal serum creatinine concentration. Renal SLE group included 19 patients (63%). They had one or more of the following: protein in urine >0.5 g 24 h^{-1}., hematuria, pyuria, urinary casts (red cell, hemoglobin, granular, tubular or mixed casts) and/or abnormal serum creatinine concentrations.

Blood sampling: Venous blood (7 mL) was obtained with informed consent from each patient and separated into three tubes; 2 mL were added to EDTA vacutainer tubes for complete blood count (CBC) in addition to erythrocyte sedimentation rate (ESR) measurement and 2 mL on heparinized vacutainer tubes for dielectric measurement, the remaining 3 mL were left to clot at room temperature for 30 min and tube was centrifuged at 1500 rpm for 15 min and serum was separated into 2 sterile aliquots; one was stored at -20°C till time of assay of VEGF and other was used for serum creatinine, ANA, anti-DNA and C_3 assay. Venous blood (3 mL) was taken from each control subject for VEGF assay and dielectric measurement.

Study measurements

- Complete blood count was performed with the (Coulter-STKS)
- Erythrocyte sedimentation rate by Westergren method.
- Complete microscopic urine analysis for WBCs, RBCs and Casts.
- Twenty-four-hour urinary total protein (Tp) by the turbidimetric method using Tp kit supplied by Stanbio (Stanbio laboratory Inc., San Antonio, Tx, USA).
- Serum creatinine was carried out on Hitachi 911 auto analyzer by Jaffe's Kinetic UV colorimetric method (Bowers *et al*., 1980).
- Serum ANA and anti-DNA by indirect immunofluorescence supplied by IMMCO diagnostics (Buffalo, NY, USA) ().
- Serum Complement component C_3 by quantitative determination using the turbidimetry (Behringwerke Diagnostics, Marburg, Germany) (Johnson., 1993).
- Lupus anticoagulant using Kits supplied by Stago Diagnostics Inc., France.
- Serum levels of VEGF in patients and controls by specific, commercially available, enzyme-linked immunosorbent assay kits (Qantikine; R and 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 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 with the range given by the manufacturer. The procedure has previously been described in detail (Robak *et al*., 2002). The concentrations of VEGF in the samples were determined by interpolation from the standard curve.
- Dielectric properties of Hb for patients and controls:

Hemoglobin preparation: The hemolysate from the washed erythrocytes was prepared by a modification of the method cited by Trivelli *et al*. (1971). The heparinized blood was centrifuged at 3500 rpm for 10 min at 4°C and then the plasma was taken out and stored at -20°C for farther investigations.

The packed red blood cells were washed with 5 volumes saline solution at 20°C and gently agitated for 2 min then re-centrifuged to separate the washing red blood cells. This step was repeated 3 times. The clean red blood cells were lysed with two volumes of deionized water. The suspension was centrifuged and Hb was separated.

Dielectric measurement: Dielectric measurements were run in the frequency range from 20 to 100 kHz using a WAYNE KERR precision component analyzer, model 6440 B(UK), connected with a conductivity cell type 19250-60 manufactured by Cole Palmer Co. The sample cell has two squared platinum black electrodes with cell constant, K = 1 cm^{-1}. The measurements were performed at 20°C. The value of the dielectric constant (ε') for the sample was calculated at each frequency from the measured values of the capacitance (C) through the equation:

$$\varepsilon' = \frac{Cd}{\varepsilon_o A}$$

where, d is the inter electrode distance in meter. A area of electrode in m^2 measured from the cell used and ε_o permittivity of free space.

The loss tangent (tan δ), the dielectric loss ε'' and AC conductivity σ were calculated from the relations

$$\text{Tan}\delta = \frac{1}{2\Pi FRC} = \frac{\varepsilon''}{\varepsilon'}$$

$$\sigma = 2\Pi F\varepsilon''\varepsilon_o$$

where, F is the frequency applied in Hz and R is the resistance of the specimen in ohms. The value of the

dielectric constant ε' falls from high value ε'_s to ε'_∞ as the frequency increases through the dispersion region where ε' the real part of the complex permittivity. The dielectric dispersion ($\Delta\varepsilon'$) was calculated by applying the relation

$$\Delta\varepsilon = \varepsilon'_s - \varepsilon_\infty$$

The average molecular radius of hemoglobin molecule was estimated from the relation

$$r^3 = \frac{kT\tau}{4\Pi\eta}$$

where, k is the Boltzmann constant, T is the absolute temperature, h is the viscosity of the Hb solution and τ the relaxation time, namely, the time at which the dielectric molecule has the ability to relax under the effect of the applied field.

The relaxation time was calculated from the equation

$$\tau = \frac{1}{2\Pi f_c}$$

where, f_c the critical frequency corresponding to the midpoint of the dispersion curve.

The Cole-Cole parameter α, which represents the correction and/or the deviation of the molecular shape from spherical assembly to non spherical, was calculated through the relation

$$\theta = \frac{\alpha\Pi}{2}$$ (Cole and Cole, 1941)

Statistical analysis: The data are represented as mean and standard deviation. Data were analyzed by the Mann-Whitney U-test. The probability of differences in frequency distributions was determined by chi-square test or Fisher's exact test. Data were correlated by Spearman's rank order test. p-values lower than 0.05 were considered statistically significant.

RESULTS

The results of this study revealed elevated serum levels of VEGF in SLE patients 501±120.5 pg mL^{-1} compared with the age-matched healthy controls 65±22.3 pg mL^{-1} ($p<0.001$) as shown in Table 1.

We investigated the correlation between serum levels of VEGF with selected clinical and laboratory findings of SLE patients. We found that SLE patients having clinical or laboratory evidence of renal involvement (renal SLE patients) had significantly higher serum levels of VEGF (640±153 pg mL^{-1}) compared with non-renal SLE patients (367±35.8 pg mL^{-1}) ($p<0.001$) as shown in Table 1.

Table 1: Serum levels of VEGF (pg mL^{-1}) in SLE patients and healthy controls

	SLE together	SLE with renal involvement	SLE without renal involvement	Healthy controls
No.	30	19	11	10
Range (Mean±SD)	501±120.5	640±153	367±35.8	65±22.3
p-value*	<0.001	<0.001	<0.001	
p-value**		<0.001		

*Statistical significance compared to VEGF level of Healthy controls, **Statistical significance compared to VEGF level of SLE without renal involvement, $p<0.05$, significant

Table 2: The dielectric increment ($\Delta\varepsilon'$), relaxation times (τ), Cole-Cole parameter (α) and average molecular radius (r) for Hb of control group and patients with SLE (non-renal and renal SLE patients)

Studied sample	Dielectric increment ($\Delta\varepsilon'$)	τ (μsec)	Alpha (α)	r (nm)
Control	3.4×10^6±0.004	2.9±0.012	0.097±0.006	3.8
Non-renal SLE patients	4.1×10^6±0.003	5.3±0.023	0.149±0.020	4.3
Renal SLE patients	6.3×10^6±0.060	8.0±0.580	0.153±0.014	4.8

Values are Means±SD

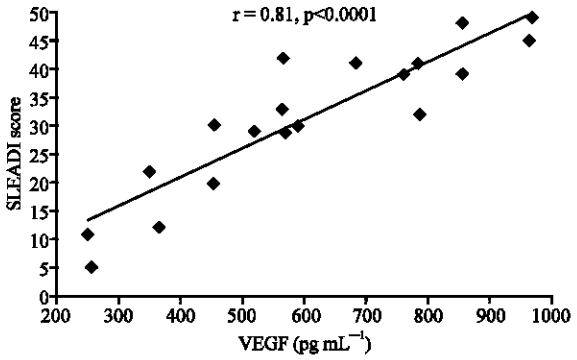

Fig. 1: Correlation between serum levels of VEGF and systemic lupus erythematosus disease activity index

Serum levels of VEGF were inversely correlated to platelet count and serum levels of C_3 ($r = -0.43$, $p<0.05$; $r = -0.59$, $p<0.001$, respectively); and correlated significantly with ESR ($r = 0.52$, $p<0.001$). A strong positive correlation was observed between the SLEDAI score and serum levels of VEGF ($r = 0.81$, $p<0.0001$) as shown in Fig. 1.

It is clear from Fig. 2 that permittivity ε' passed through a dielectric dispersion in the frequency range demonstrated and the decrease in the values of ε' was accompanied by an increase in the values of conductivity o, which is a confidence in dielectric measurements (Grant, 1983). This strong dielectric dispersion in the frequency range of 20 to 100 kHz for samples is mainly due to protein and counter ion molecular relaxation (Polk and Postow, 1996). It is clear from results of Table 2 that there are some changes in values

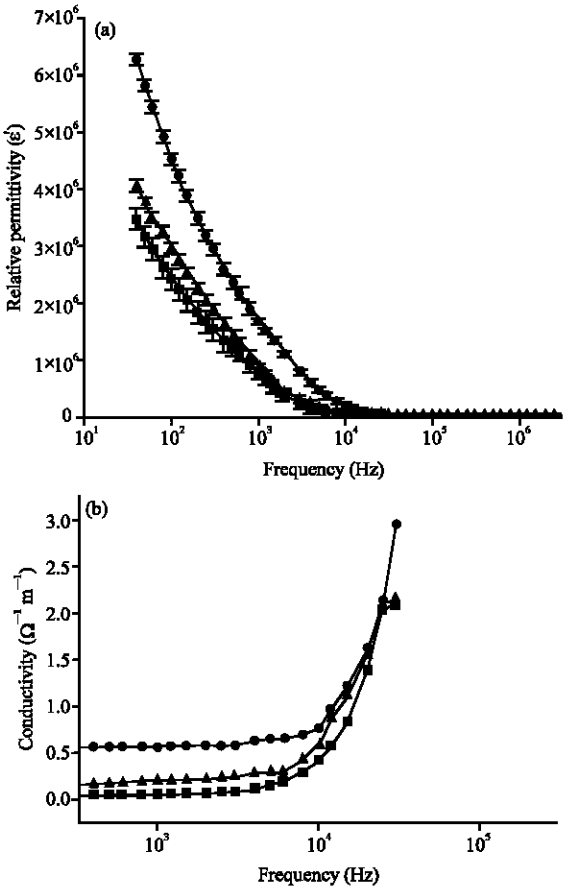

Fig. 2: Variation of relative permittivity ε′ (a) and conductivity o (b) as a function of the applied frequency in the range of 20 Hz to 100 kHz for Hb collected from healthy persons control (■), non-renal SLE patients (▲) and renal SLE patients (●)

of (Δε′), (τ) and (α) for Hb from renal SLE patients as compared with non-renal SLE patients and control.

DISCUSSION

Many studies have concentrated on the role of angiogenesis and microvascular endothelial injury in the pathogenesis of systemic organ involvement in rheumatic diseases, such as SLE (Clancy *et al*., 2001). One of the key players in the process of new vessel formation is VEGF (Ferrara, 1999; Paleolog, 1996). Vascular conditions such as those occurring in SLE, which include inflammation, vessel occlusion or thickening of vascular wall might be a strong stimulus for angiogenic factor production (Plate and Warnke, 1997; Thomas *et al*., 1996; Blau and Banfi, 2001). In the present study, we analyzed serum levels of VEGF in patients with SLE. We found elevated levels of VEGF in SLE patients when compared with healthy controls. Some investigators reported results similar to ours (Robak *et al*., 2002; Navarro *et al*., 2002; Robak *et al*., 2003), whereas others observed comparable serum VEGF values in SLE patients and controls (Kikuchi *et al*., 1998). In addition, we tried to investigate the association between VEGF and clinical feature, laboratory findings as well as SLE activity.

The results of the current study showed that VEGF serum levels were positively correlated with SLEDAI score (Fig. 1). Moreover, VEGF reached the highest serum levels in patients with renal involvement. The results revealed that VEGF was significantly higher in SLE patients with renal involvement compared to non-renal SLE patients. It was reported that urinary VEGF mRNA levels are increased in active nephritis and are decreased with the response to treatment (Avihingsanon *et al*., 2006). In normal human kidney, VEGF mRNA and protein are pre-dominantly strongly expressed by visceral glomerular epithelial cells (podocytes), where its physiological function and role in development of renal disease are obscure and uncertain (Brown *et al*., 1992; Shulman *et al*., 1996; Honkanen *et al*., 2000). In many glomerular diseases, VEGF expressing cells were decreased in number or absent in areas of focal or global glomerular sclerosis. The decrease in the number of VEGF-expressing cells noted in glomerular diseases is likely a result of epithelial damage or destruction. Damage of visceral cells in a variety of glomerular diseases has the potential for releasing relatively large amounts of VEGF locally, leading to increased glomerular permeability and alterations. In addition, loss of normal controlled secretion of VEGF after damage to visceral epithelial cells could lead to important alteration in glomerular endothelial cell function (Shulman *et al*., 1996). VEGF was found to mediate glomerular endothelial repair promoting healing from glomerular injury and it was suggested as a novel therapeutic approach to glomerular diseases characterized by endothelial damage, such as various glomerulonephritides and renal transplant rejection (Iruel-Arispe *et al*., 1995; Noguchi *et al*., 1998; Ostendorf *et al*., 1999). Other investigators reported that patients with renal failure had significantly elevated plasma levels and over expression of VEGF in renal tissue compared with SLE patients with normal renal function and it is postulated that this factor might be involved in glomerular endothelial repair (Navarro *et al*., 2002). This observation, in addition to the increased serum VEGF detected in our renal SLE patients, makes it possible that VEGF is related to endothelial repair in SLE. However the role of VEGF in pathogenesis of lupus nephritis or in its healing is in need of further studies to be elucidated.

Present results for ε′ of Hb molecules indicated a strong dielectric dispersion (Fig. 2). The dielectric behavior showed an anomalous frequency dispersion as it was found in different biological materials (Gabriel *et al*., 1996; Ghannam *et al*., 2002; Foster and Schwan, 1995). Moreover ε′ and σ of the Hb from SLE patients group were higher than their counter parts of the control group. It is very well established that the magnitude of dielectric constant ε′ is due to counter-ions polarization of protein molecules (Polk, 1992).

These molecules are impeded and surrounding the cell membrane which serves as receptors of chemical signals and also as detectors of electric and mechanical disturbance (Campbell, 1996). The change of dielectric increment (Δε′), relaxation times (τ, Cole-Cole parameter (α) and molecular radius (r) for Hb of SLE patients as compared with control (Table 2) indicates that some changes in the molecular structure occurred for Hb from SLE patients(non- renal and renal). The Hb molecules had changed from spherical shape for control to non spherical like shape for SLE patients (non-renal and renal). The radius of the molecules was large for SLE patients as compared with control. The expected reason for these changes may be vascular conditions which occurring in SLE such as inflammation, vessel occlusion and/or thickening of the vascular wall, these conditions may change the ion transport through the membrane. Also the radius of molecule was large for renal SLE group as compared with non-renal group, this may be due to the role of uremic toxins which have increasing concentration in blood serum, it can interact with RBCs membrane phospholipid and affects the membrane ionic transport leading to the measured changes in shapes and diameter (Ibrahim *et al*., 2002). One more point is that SLE and uremia causes the changes in the counter ion molecules of the RBCs and hence strongly affects the cell communication, which will be directly reflected on the cellular metabolic activities.

CONCLUSION

In conclusion, VEGF serum levels were increased in SLE patients. It was significantly higher in patients with renal involvement than those without. A strong positive correlation was demonstrated between serum levels of VEGF and the SLEDAI score, suggesting the possibilities of its use as a monitor for SLE disease activity. This research also indicated that the measurements of dielectric properties of Hb in SLE patients may be a good supplementary study to explain the role of angiogenesis in the pathogenesis of SLE. Better understanding of the pathogenesis may provide novel approach for treating SLE patients.

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