Elevated Vascular Endothelial Growth Factor in Systemic Sclerosis

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ABSTRACT. ***Objective.*** To determine the serum levels of vascular endothelial growth factor (VEGF) in patients with systemic sclerosis (SSc) and to search for relationships between its serum levels and the clinical manifestations.

Methods. Serum levels of VEGF in patients with SSc and healthy controls were determined by ELISA. At the time of blood sampling, individual organ involvement was assessed, and a video microscope and PC based image processing were used to visualize nailfold capillaries and to quantify capillary density.

Results. Serum levels of VEGF in 48 patients with SSc were significantly higher than in 30 controls (432 ± 356 vs 91 ± 64 pg/ml; $p < 0.001$). Patients with diffuse cutaneous SSc (n = 21) had higher levels of serum VEGF than those with limited cutaneous SSc (n = 27) (432 ± 356 vs 135 ± 127 pg/ml; $p < 0.001$). Serum VEGF levels correlated well with the extent of skin sclerosis, as determined by modified Rodnan skin score ($r = 0.656$, $p < 0.001$) and serum TGF-ß levels ($r = 0.530$, $p < 0.001$). In particular, serum VEGF levels were inversely correlated with the capillary density of nailfold ($r = –0.649$, $p < 0.001$). However, no significant differences were found in the serum levels of VEGF between patients with systemic organ involvement and those without.

Conclusion. The extent of skin sclerosis may contribute to the elevation of serum VEGF and high VEGF levels may serve as a surrogate indicator of capillary damage in SSc. (J Rheumatol 2003;30:1529–33)

Key Indexing Terms:
SYSTEMIC SCLEROSIS VASCULAR ENDOTHELIALGROWTH FACTOR
RODNAN SKIN SCORE CAPILLARY DENSITY ANGIOGENESIS
NAILFOLD CAPILLARY MICROSCOPY

Systemic sclerosis (SSc) is a disease of connective tissue, and is characterized by fibrosis and vascular damage in the skin and various visceral organs[1]. The pathogenesis of SSc remains unknown, but vascular alterations have been suggested to play an important role in this disease[2]. The vascular alterations in SSc are damage and activation of endothelial cells, with resultant blood flow disturbance. Microvascular involvement usually precedes the development of skin sclerosis, and its clinical manifestation includes Raynaud's phenomenon, early edematous features, telangiectasia, and digital ulcers. The vascular changes recognized by nailfold capillary microscopy (NCM) are characterized by distorted capillary loops and reduced numbers of capillaries, leading to decreased capillary density and avascular areas[3,4].

Vascular endothelial growth factor (VEGF) is a 45 kDa heparin-binding glycoprotein that induces the proliferation and migration of endothelial cells to form new vessels, and increases vascular permeability[5,6]. VEGF is produced by endothelial cells, smooth muscle cells, fibroblasts, macrophages, and a variety of other cell types[6-11], when exposed to hypoxic conditions or stimulated with interleukin 1 (IL-1), IL-6, transforming growth factor-ß (TGF-ß), or CD40 ligand[7,8,12-14]. VEGF expression is increased in several pathologic conditions including atherosclerosis, ischemic heart disease, diabetic retinopathy, inflammatory diseases, and tumor formation[5,15]. Kikuchi, *et al*[16] reported high levels of VEGF in several connective tissue diseases including SSc. Our aim was to determine the serum levels of VEGF, and to investigate the relationship between these and clinical features such as skin sclerosis, digital capillary loss, and organ involvement, in patients with SSc.

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MATERIALS AND METHODS

Patients. Serum samples were collected from 48 Korean patients with SSc (45 women and 3 men) at Kangnam St. Mary's Hospital over a period of 2 years. All patients fulfilled criteria of the American College of Rheumatology for SSc[17]. Twenty-one of the 48 patients had diffuse cutaneous SSc (dSSc) and 27 patients limited cutaneous SSc (lSSc), according to the guidelines of LeRoy, *et al*[18]. The mean age of the SSc patients was 40.6 years (range 20–72). Mean disease duration was 6.9 years when defined as duration from onset of Raynaud's phenomenon. Most patients had received medications including D-penicillamine, prednisolone, or colchicine, but all medications were stopped 48 h before blood sampling to minimize the effects of medications. Comparisons were made with 30 healthy controls (all female, mean age 38 yrs, range 24–48) with no symptoms of rheumatic diseases. No difference was found in age and sex between SSc patients and healthy controls.

Clinical and laboratory evaluation. Clinical and laboratory assessments were done at the time of blood sampling, and included age, sex, disease duration, type of SSc, modified Rodnan score[19], and presence of organ involvement. Esophageal involvement was assessed by endoscopy and esophageal manometry. Pulmonary interstitial fibrosis was defined as bibasilar interstitial fibrosis on chest radiographs and, in patients with no abnormalities on chest radiographs, as presence of alveolitis on high resolution computerized tomography (HRCT). Pulmonary hypertension was assessed by echocardiogram, and cardiac involvement was defined as any of the following: symptomatic pericarditis, clinical evidence of left ventricular congestive heart failure, or arrhythmias requiring treatment. Joint involvement was defined as inflammatory polyarthralgia or arthritis. Laboratory variables including complete blood count, urinalysis, C-reactive protein (CRP) levels, erythrocyte sedimentation rate (ESR), and antibodies to topoisomerase I and centromere-B were assessed.

Nailfold capillary microscopy. Skin capillaries were observed and photographed in all patients using a stereozoom microscope (Olympus SZ-PT) at a magnification of 30×. Immersion oil was placed on the patient's nailfold bed to enhance its transparency, and temperature was controlled by ensuring that every subject was indoors for a minimum of 15 min before the nailfolds were examined. Images of the center of the nailfold were displayed on a color monitor and printed.

The number of capillaries in the distal row within 3 mm of the center of the nailfold was counted on the third and fourth digits of each hand, and the mean of the left and right 4 digits is presented. In the controls, right digits were examined, as we observed that there were no significant differences in capillary density between the left and right digits of controls (data not shown). During nailfold capillaroscopic analysis, enlargement of capillary loops and loss of capillaries[20] were considered as the most specific characteristic features of SSc, although other alterations were also evaluated, i.e., giant capillaries, hemorrhages, ramified/bushy capillaries, and pearl formation ("white band" observed in SSc patient's nailfolds between the end row capillaries and the cuticle).

Determination of VEGF and TGF-ß concentrations. Serum VEGF from patients with SSc and healthy subjects was measured by sandwich ELISA[14]. Ninety-six-well microtiter plates were coated with 100 µl per well of 0.4 µg/ml goat anti-human $VEGF_{165}$ (R & D Systems, Minneapolis, MN, USA) buffered with 50 mM of sodium carbonate (pH 9.6). After incubation overnight at 4°C, the plates were blocked with 1% bovine serum albumin in phosphate buffered saline (PBS) for 1 h at room temperature. Human recombinant $VEGF_{165}$ (R & D Systems) or test samples were added to the wells and then reacted with the plate for 2 h at room temperature. The plates were incubated with 0.2 µg/ml biotinylated goat anti-human $VEGF_{165}$ (R & D Systems) at room temperature for 2 h. Peroxidase-labeled extravidin (Sigma Bioscience, St. Louis, MO, USA), diluted 1:1000, was then added to the wells and reacted at room temperature for 1 h. The color reaction was induced by the addition of substrate solution (tetramethylbenzidine/hydrogen peroxide) and was stopped 30 min later by the addition of 1 M phosphoric acid. An automated microplate reader was used to measure the OD at a wavelength of 450 nm. Between each of these steps, the plates were washed 4 times with PBS containing 0.1% Tween 20. Human recombinant $VEGF_{165}$ diluted in culture medium was used as a calibration standard, at concentrations ranging from 10 to 2000 pg/ml. A standard curve was drawn by plotting OD versus the log of the recombinant $VEGF_{165}$ concentration. The values above the mean + 2 standard deviations (SD) of the healthy controls were considered to be elevated. Circulating TGF-ß was measured in the same samples using sandwich ELISA, as described[21].

Statistical analysis. Statistical significance of the difference between groups was determined using the Mann-Whitney U test and the relations between variables were calculated using Spearman's rank correlation. Results were considered statistically significant with corresponding 2 sided $p < 0.05$.

RESULTS

The serum levels of VEGF in 48 SSc patients were significantly higher than in 30 healthy controls (432 ± 356 vs 91 ± 64 pg/ml; $p < 0.001$, Figure 1). Among the SSc subsets, serum VEGF levels were significantly higher in the patients with dSSc than in the patients with lSSc (432 ± 356 vs 135 ± 127 pg/ml; $p < 0.001$; Figure 1). Serum levels of VEGF were higher in patients with lSSc compared to healthy controls ($p = 0.037$). When values above the mean + 2 SD of the healthy controls were considered to be elevated, patients with elevated VEGF levels were found in 17 of 48 patients with SSc (35%). In addition, the frequency of elevated VEGF levels in patients with dSSc was significantly higher than that in patients with lSSc (71% vs 7%; $p < 0.001$).

We compared the clinical and laboratory features of SSc patients with elevated and normal VEGF levels. Table 1 shows that patients with elevated VEGF levels have higher Rodnan skin scores than those with normal VEGF levels. In particular, serum VEGF levels correlated well with the extent of skin sclerosis as determined by the modified Rodnan skin score ($r = 0.656$, $p < 0.001$; Figure 2A) and the serum levels of TGF-ß ($r = 0.530$, $p < 0.001$; Figure 2B). However, there was no correlation between VEGF levels and ESR or CRPlevels. Patients with elevated VEGF levels tended to have longer disease duration than those with normal VEGF levels, but it did not reach statistical significance. There were no differences in the other clinical features or the medications used between patients with elevated and normal VEGF levels.

Next, we analyzed nailfold capillary densities by capillary microscope to determine whether increased levels of VEGF are associated with capillary damage in SSc. The nailfold capillary density (total number of capillary loops in 3 mm) was significantly lower in SSc patients than in controls (14.6 ± 4.4 vs 20.6 ± 2.3; $p < 0.001$), and the number of capillary loops in patients with elevated VEGF levels was significantly lower than in the patients with normal VEGF levels (10.4 ± 3.4 vs 17.0 ± 2.9; $p < 0.001$; Table 1). In particular, serum VEGF levels in SSc patients were inversely correlated with the total number of capillary loops ($r = –0.649$, $p < 0.001$; Figure 3).

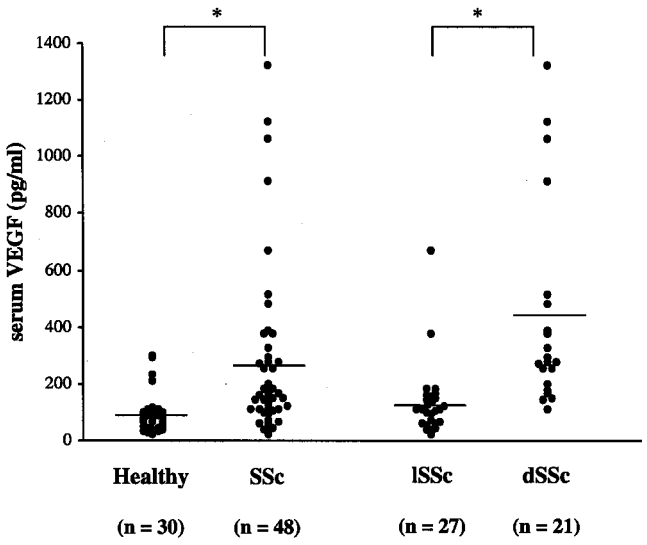

Figure 1. Serum levels of vascular endothelial growth factor (VEGF) in healthy controls and patients with SSc; dSSc: diffuse cutaneous; lSSc: limited cutaneous SSc. Horizontal bars represent mean values. *p < 0.001.

We demonstrated that patients with SSc had significantly higher serum VEGF levels than healthy controls, and VEGF levels were significantly higher in dSSc patients than in lSSc patients. The study also showed that VEGF correlated with the extent of skin sclerosis and serum TGF-ß levels. In particular, we observed a positive correlation between the serum levels of VEGF and the severity of nailfold capillary loss.

In SSc, fibroblasts from dermal tissue are known to exhibit the increased expression of intercellular adhesion molecule I, MHC class II molecules, and the enhanced transcription of collagen genes[26-28], and production of cytokines such as IL-6 and monocyte chemoattractant protein-1[29-31], which suggests that dermal fibroblasts are in an activated state. The capability of activated fibroblasts derived from keloid tissues to produce VEGF has been well documented[32]. In our study, serum levels of VEGF correlated with the extent of skin sclerosis; it therefore seems to be possible that elevated VEGF in serum originates from activated fibroblasts of skin.

Table 1. Comparison of clinical features between systemic sclerosis patients with elevated VEGF levels and those with normal VEGF levels.

	Elevated VEGF, n = 17	Normal VEGF, n = 31	p
Age of patients, yrs*	38.2 ± 12.0	41.9 ± 12.9	NS
Sex, M:F	2:15	1:30	NS
Disease duration, yrs*	8.8 ± 6.4	5.8 ± 5.1	0.096
Organ involvement, (%)			
Esophagus	10 (59)	11 (35)	NS
Pulmonary interstitial fibrosis	9 (53)	16 (52)	NS
Heart	4 (24)	3 (10)	NS
Joint	10 (59)	16 (52)	NS
Anti-topoisomerase, n (%)	9/17 (53)	12/29 (41)	NS
Rodnan skin score	18.5 ± 7.8	9.4 ± 6.6	0.001
Nailfold capillary density*†	14.6 ± 4.4	20.6 ± 2.3	< 0.001

* Mean ± SD. † Total number of capillary loops in 3 mm length of the nailfold center. NS: not significant.

DISCUSSION

Several studies have reported discrepant angiogenic potentials in SSc. The angiogenic capability of SSc lymphocytes was found to be low compared with that of controls when injected intradermally into x-ray immunosuppressed mice[22]. Koch, *et al* demonstrated that the conditioned medium of lipopolysaccharide-stimulated SSc monocytes showed significantly lower angiogenic potential in a rat corneal bioassay[23]. In contrast, sera from early limited SSc patients exhibited enhanced angiogenic ability[24], and a monocyte-enriched population from SSc patients exhibited increased angiogenesis in a xenogenic system[25]. Such discrepancies might be explained by the different samples (i.e., the sera and cell types used) and the methods of measurement adopted in these studies.

Microvascular damage in SSc is characterized by structural alterations of capillaries that lead to a progressive decrease in capillary density and insufficient blood perfusion to many organ systems, and ultimately to chronic ischemia[33]. Figure 3 shows that capillary density as assessed by nailfold capillary microscopy is inversely correlated with VEGF level. This finding suggests that increased level of VEGF may be secondary to microvascular damage and resultant tissue hypoxia. In view of the fact that VEGF is a potent inducer of endothelial cell proliferation and angiogenesis, this result might not explain the capillary loss observed in SSc. In fact, endothelial cell response to hypoxic stress can be varied, depending on the duration of hypoxia. Hypoxia is known to induce a variety of genes including platelet derived growth factor-B, insulin-like

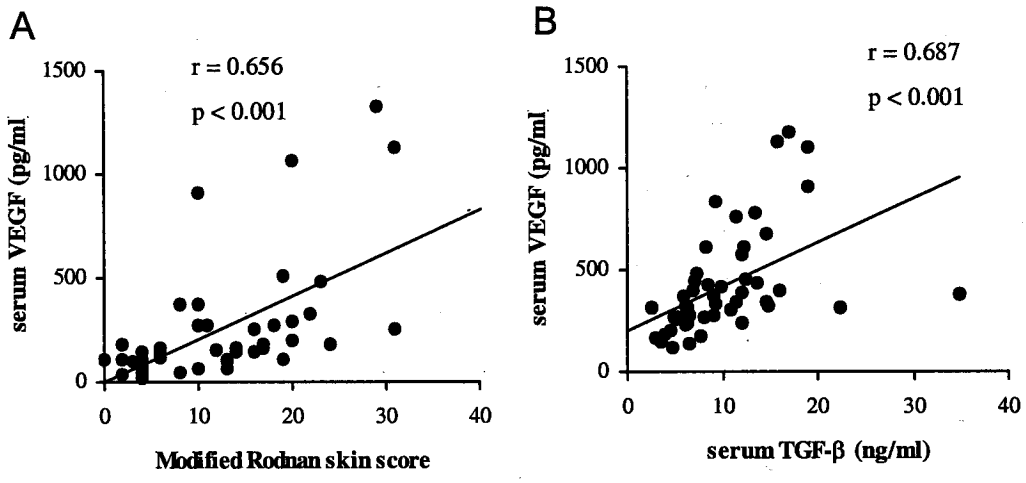

Figure 2. Correlations between serum VEGF levels and modified Rodnan skin score (A) or serum transforming growth factor-ß (TGF-ß) levels (B) in patients with SSc.

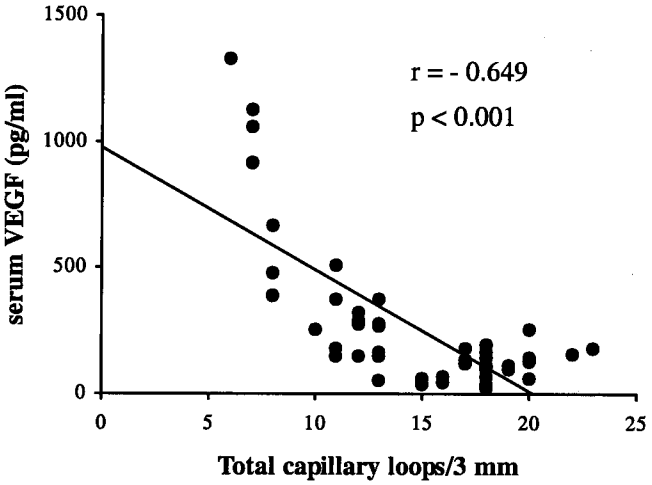

Figure 3. Inverse correlations of serum VEGF levels with capillary density (total capillary loops/3 mm) as determined by nailfold capillary microscopy.

growth factor, basic fibroblast growth factor, endothelin-1, and VEGF[34]. These gene products, in turn, mediate irreversible structural remodeling of the vasculature and the surrounding tissues by acting as mitogens for endothelial or smooth muscle cells and fibroblasts, thus leading to more fibrosis and hypoxia. In addition to tissue hypoxia, TGF-ß is a well known angiogenic factor that can stimulate VEGF production in several cell types. We found a positive correlation between serum levels of VEGF and TGF-ß. Collectively, it appears that VEGF can be induced in response to chronic hypoxia caused by microvascular damage and by TGF stimulation.

On the other hand, new capillaries are rarely seen and broad avascular areas are common in SSc, even at high concentrations of VEGF, which indicates possible defects in critical elements of angiogenesis. This finding is analogous to that of rheumatoid synovitis. In inflamed synovium vascular densities are not increased despite the enhanced production of angiogenic factors, thus showing an anaerobic metabolism[35-37]. Microvascular toxicity by oxidative stress, tumor necrosis factor-α, and angiotensin II[38-40], which are increased in SSc, might contribute to the defective angiogenesis of SSc. In addition, Hebbar, *et al*[41] demonstrated that increased concentrations of the angiogenesis inhibitor endostatin were observed in patients with SSc, and were associated with digital gangrene. These results indicate that the imbalance between angiogenic and anti-angiogenic factors also explains the capillary loss in SSc, even in the presence of high VEGF levels.

In conclusion, serum VEGF levels in SSc were enhanced and correlated well with the extent of skin sclerosis and serum TGF-ß levels. Further, the levels of VEGF were inversely associated with capillary density in SSc. These findings suggest that skin sclerosis might contribute to the elevation of serum VEGF, and that serum VEGF may be a surrogate indicator of capillary damage in SSc. The underlying mechanism of impaired angiogenesis even at high VEGF concentrations remains to be determined.

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