Research article

Angiogenic and angiostatic factors in systemic sclerosis: increased levels of vascular endothelial growth factor are a feature of the earliest disease stages and are associated with the absence of fingertip ulcers

Oliver Distler[1], Angela del Rosso[2], Roberto Giacomelli[3], Paola Cipriani[3], Maria L Conforti[2], Serena Guiducci[2], Renate E Gay[1], Beat A Michel[1], Pius Brühlmann[1], Ulf Müller-Ladner[4], Steffen Gay[1] and Marco Matucci-Cerinic[2]

[1]Center of Experimental Rheumatology, Department of Rheumatology, University Hospital Zurich, Switzerland
[2]Department of Medicine, Section of Rheumatology, University of Florence, Italy
[3]Department of Internal Medicine and Public Health, University of L'Aquila, Italy
[4]Department of Internal Medicine I, University of Regensburg, Germany

Corresponding author: Steffen Gay (e-mail: Steffen.Gay@ruz.usz.ch)

Received: 14 May 2002 Revisions received: 30 July 2002 Accepted: 6 August 2002 Published: 30 August 2002

Arthritis Res 2002, **4**:R11 (DOI 10.1186/ar596)

Abstract

To examine whether the lack of sufficient neoangiogenesis in systemic sclerosis (SSc) is caused by a decrease in angiogenic factors and/or an increase in angiostatic factors, the potent proangiogenic molecules vascular endothelial growth factor (VEGF) and basic fibroblast growth factor, and the angiostatic factor endostatin were determined in patients with SSc and in healthy controls. Forty-three patients with established SSc and nine patients with pre-SSc were included in the study. Serum levels of VEGF, basic fibroblast growth factor and endostatin were measured by ELISA. Age-matched and sex-matched healthy volunteers were used as controls. Highly significant differences were found in serum levels of VEGF between SSc patients and healthy controls, whereas no differences could be detected for endostatin and basic fibroblast growth factor. Significantly higher levels of VEGF were detected in patients with Scl-70 autoantibodies and in patients with diffuse SSc. Patients with pre-SSc and short disease duration showed significant higher levels of VEGF than healthy controls, indicating that elevated serum levels of VEGF are a feature of the earliest disease stages. Patients without fingertip ulcers were found to have higher levels of VEGF than patients with fingertip ulcers. Levels of endostatin were associated with the presence of giant capillaries in nailfold capillaroscopy, but not with any other clinical parameter. The results show that the concentration of VEGF is already increased in the serum of SSc patients at the earliest stages of the disease. VEGF appears to be protective against ischemic manifestations when concentrations of VEGF exceed a certain threshold level.

Keywords: basic fibroblast growth factor, endostatin, fingertip ulcers, systemic sclerosis, vascular endothelial growth factor

Introduction

Systemic sclerosis (SSc) is a generalized fibrotic connective tissue disease that affects the skin and various internal organs. Histopathological hallmarks of SSc are perivascular infiltrates and a reduced capillary density, which precede the excessive accumulation of extracellular matrix proteins in the later stages of the disease [1]. The reduced capillary density leads to a reduced blood flow, to tissue ischemia and to clinical manifestations such as fingertip ulcers [2]. Tissue hypoxia usually initiates the formation of new blood vessels from the pre-existing microvasculature. Despite the reduced blood flow and reduced partial oxygen pressure levels, there is paradoxically no evidence for a sufficient angiogenesis in the skin of patients with SSc [3].

Angiogenesis is a complex multistep process that is under the tight control of angiogenesis inducers and inhibitors. Under normal conditions, the levels of angiogenesis

bFGF = basic fibroblast growth factor; ELISA = enzyme-linked immunosorbent assay; SSc = systemic sclerosis; VEGF = vascular endothelial growth factor.

inducers and inhibitors are balanced and angiogenesis does not occur in healthy tissues. In a hypoxic environment and in inflammatory states such as rheumatoid arthritis, angiogenic growth factors are induced and outweigh the inhibitors, resulting in the initiation of angiogenesis [4].

Among the angiogenesis inducers, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) have been characterized as key molecules in the induction of angiogenesis. VEGF is involved in several steps of physiological and pathological angiogenesis including proliferation, survival and migration of endothelial cells. The biological effects of VEGF are extremely dose dependent. Loss of even a single allele results in lethal vascular defects in the embryo, and postnatal inhibition of VEGF leads to impaired organ development and growth arrest in mice [5–7]. Application of VEGF as a recombinant protein or by gene transfer augmented perfusion and development of collateral vessels in animal models of hindlimb ischemia, thereby making VEGF an interesting target for therapeutic angiogenesis [8,9].

In contrast to VEGF, genetic loss of bFGF does not cause major vascular defects and bFGF has no exclusive specificity for endothelial cells. However, bFGF has been shown to stimulate proliferation, migration and differentiation of endothelial cells and it synergies potently with VEGF in its angiogenic actions. Similar to VEGF, bFGF stimulates angiogenesis in different animal models for ischemic diseases [10,11].

Endostatin is a C-terminal, 20 kDa fragment of the basement protein collagen type XVIII. Endostatin inhibits angiogenesis and tumor growth strongly by reducing endothelial cell proliferation and migration [12]. Recent data suggest that cathepsin L is involved in the cleavage of endogenous endostatin from perivascular collagen type XVIII [13]. Although the mechanisms of action are not fully elucidated, it has been shown that endostatin inhibits the proteolytic activation of pro-matrix metalloproteinase-2 and the catalytic activities of membrane type 1 matrix metalloproteinase and matrix metalloproteinase-2 [14].

Angiogenesis is strongly disturbed in SSc, as demonstrated by nailfold capillaroscopy changes. Capillary dropouts can often be found in later stages of the disease. Before this endpoint, however, angiogenesis appears to be disturbed at different levels, and a variety of morphological changes can be detected (e.g. megacapillaries, bushy capillaries). The modification of the angiogenic process is thus contributing to the chronically reduced oxygen supply of the tissue, resulting in ischemic manifestations such as fingertip ulcers [15].

The lack of a sufficient response to hypoxia and other stimuli to form functional vessels in patients with SSc might be explained by an inappropriate synthesis of angiogenic factors or an inhibition by angiostatic factors. The aim of the present study was to analyze whether a decrease of the angiogenic factors VEGF and bFGF and an increase of the angiostatic factor endostatin contribute to the impaired angiogenesis in patients with SSc, and whether these factors may correlate with the main clinical features and parameters of vascular involvement.

Materials and methods

Patients

Forty-three consecutive patients with SSc were recruited at the Section of Rheumatology of the University of Florence. All patients fulfilled the American College of Rheumatology criteria for SSc [16]. There were 35 women and eight men with a median age of 61 years (range, 24–79 years). Patients with overlap symptoms to other connective tissue diseases were excluded from the study.

Nine patients without skin involvement were included to assess the levels of angiogenesis-related molecules in a prescleroderma condition [17]. These patients presented with Raynaud's phenomenon, nailfold capillaroscopy changes and circulating autoantibodies characteristic for SSc (anti-topoisomerase I, anticentromere or antinuclear with nucleolar pattern). The nine pre-SSc patients were all female, with a median age of 58 years (range, 32–70 years).

Healthy volunteers ($n = 21$) were used as controls. The control group consisted of 16 women and five men with a median age of 55 years (range, 29–96 years). An additional control group ($n = 20$) was used for the endostatin measurements, consisting of 17 women and three men with a median age of 49 years (range, 23–69 years). All patients and controls were of Caucasian origin.

Clinical assessment

An extensive clinical profile was established for each pre-SSc patient and each SSc patient. Patients' characteristics are summarized in Table 1.

SSc patients were classified as affected by limited SSc or by diffuse SSc according to the criteria proposed by LeRoy *et al.* [18]. Disease stages were defined as suggested by Medsger and Steen [19]: early limited SSc, disease duration <5 years; intermediate/late limited SSc, disease duration ≥ 5 years; early diffuse SSc, disease duration <3 years; and intermediate/late SSc, disease duration ≥ 3 years.

The presence of fingertip ulcers at the time of blood drawing, other skin ulcers (e.g. at the lower extremities, elbows, forearms), teleangiectasias and disease duration since first nonRaynaud symptoms were recorded. All patients reported the occurrence of Raynaud's phenomenon after exposure to low temperatures. The modified

Table 1

Clinical characteristics of systemic sclerosis (SSc) patients, patients with pre-SSc and healthy controls

Characteristic	SSc ($n = 43$)	Pre-SSc ($n = 9$)	Healthy ($n = 21$)
Age (years), median (range)	61 (24–79)	58 (32–70)	55 (29–96)
Gender			
Male	8/43	0/9	5/21
Female	35/43	9/9	16/21
Disease subset			
Diffuse	23/43	–	
Limited	20/43	–	
Disease phase			
Early	25/43	–	
Intermediate/late	18/43	–	
Fingertip ulcers			
Positive	16/43	–	
Negative	27/43	–	
Other skin ulcers			
Positive	18/43	–	
Negative	25/43	–	
Skin score			
Diffuse SSc, median (range)	22 (4–45)	–	
Limited SSc, median (range)	11 (4–30)	–	
Capillaroscopy			
Early	6/43	1/9	
Active	22/43	7/9	
Late	14/43	0/9	
No changes	1/43	1/9	
Autoantibodies			
Antinuclear antibody-positive	39/43	9/9	
Anti-Scl-70 autoantibody-positive	13/43	0/9	
Anticentromere antibody-positive	11/43	7/9	
No autoantibodies	4/43	0/9	
Carbon monoxide diffusion capacity (%), median (range)	70 (26–144)	–	

See text for definitions.

Rodnan skin score was assessed by an experienced rheumatologist at 17 body areas by clinical palpation and was rated 0–3, with a maximum total score of 51 [20].

Nailfold videocapillaroscopy was performed in a blinded manner for the analysis of microvascular abnormalities. Patients were allowed to adapt to room temperature (20–22°C) for at least 15 min before the examination was started. The nailfolds of all 10 fingers were analyzed for the following parameters: presence of enlarged and giant capillaries, pericapillary edema, hemorrhages, loss of capillaries, ramified/bushy capillaries and disorganization of the vascular distribution.

According to these analyzed features, patients were grouped into capillaroscopy changes with an early, active and late pattern using the criteria proposed by Cutolo *et al*. [21]. The early pattern included the criteria of few giant capillaries and capillary hemorrhages, relatively well preserved capillary distribution and no evident loss of capillaries. The criteria for the active pattern were frequent capillary hemorrhages and giant capillaries, moderate loss of capillaries with some avascular areas, mild disorganization of the capillary architecture and absent or some ramified capillaries. Finally, the late pattern criteria were irregular enlargement of capillaries, few or absent giant capillaries, absence of hemorrhages, severe loss of capillaries with large avascular areas, severe disorganization of the normal capillary distribution and frequent ramified/bushy capillaries.

Pulmonary involvement was examined by the carbon monoxide diffusion capacity using the single-breath method standardized for hemoglobin.

Antinuclear antibodies were determined by ELISA, anticentromere antibodies determined on Hep-2 cells and anti-topoisomerase I (Scl-70) antibodies were determined by immunoblot analysis.

Concomitant treatment of SSc patients included angiotensin-converting enzyme inhibitors, calcium channel blockers, proton-pump inhibitors, clebopride and topical glyceryl trinitrate. Patients with pre-SSc were treated with calcium channel blockers and topical glyceryl trinitrate. All patients had received therapy with intravenous prostanoids. None of the study patients received corticosteroids, methotrexate, cyclophosphamide, D-penicilliamine or other potentially disease-modifying drugs.

ELISA for VEGF, bFGF and endostatin

After signed consent, blood samples were drawn from patients as well as from healthy controls from the antecubital vein, between 8:00 and 9:00 a.m. Samples were centrifuged, and the obtained sera were stored in aliquots at –20°C until analyses.

Levels of VEGF and bFGF protein were determined by quantitative colorimetric sandwich ELISA (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. Concentrations were calculated using a standard curve generated with specific standards provided by the manufacturer.

The ELISA for VEGF recognizes human $VEGF_{165}$ with crossreactivity to $VEGF_{121}$, but not to factors related to VEGF such as human placental growth factor and platelet-derived growth factor. Interassay and intra-assay variances were less than 10%. The minimum detectable concentration of VEGF was less than 9.0 pg/ml.

The assay for bFGF showed minimal crossreactivity to FGF-4 (0.02%), but not to other factors related to bFGF such as acidic fibroblast growth factor. The minimal detectable concentration of bFGF in serum was less than 3 pg/ml, and the interassay and intra-assay variances were less than 10%.

The serum levels of the free form of human endostatin were determined by quantitative colorimetric sandwich ELISA (CytElisa Human Endostatin; CytImmune, College Park, MD, USA) according to the manufacturer's instructions. Concentrations were calculated using a standard curve generated with specific standards provided by the manufacturer. The assay did not show any crossreactivity with World Health Organization standards for any human and murine cytokine, including murine endostatin. The minimum detectable concentration of endostatin was less than 12 pg/ml, and the interassay and intra-assay variances were less than 10%.

Statistical analysis

Data are shown as box plots with median and upper and lower quartiles if not otherwise indicated. The Kruskal–Wallis test was used for analysis of differences between more than two groups, and the Mann–Whitney test was used for subanalysis between two specific groups. For comparison of continuous variables, the Spearman's rank test was applied. $P < 0.05$ was considered of statistical significance.

Results

Circulating levels of VEGF

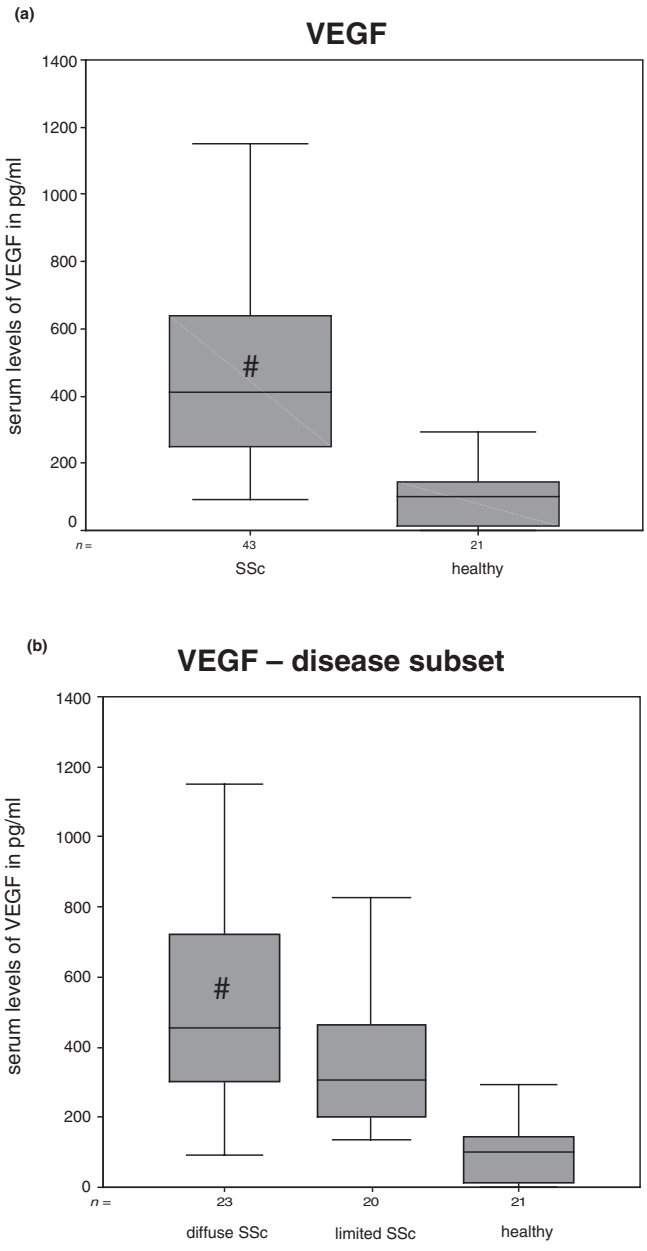
Highly significant differences were found in serum levels of VEGF between patients with SSc (median, 412 pg/ml; range, 93–1151 pg/ml) and age-matched and sex-matched healthy controls (median, 101 pg/ml; range, not detectable–377 pg/ml; $P < 0.001$), as illustrated in Fig. 1a.

When analyzed according to the disease subset, both patients with diffuse SSc as well as patients with limited SSc showed significantly increased levels of VEGF compared with healthy controls ($P < 0.001$) (Fig. 1b). In addition, patients with diffuse disease (median, 442 pg/ml; range, 93–1151 pg/ml) showed significantly higher levels of VEGF than patients with limited disease (median, 283 pg/ml; range, 135–826 pg/ml; $P \leq 0.02$).

Circulating levels of endostatin and bFGF

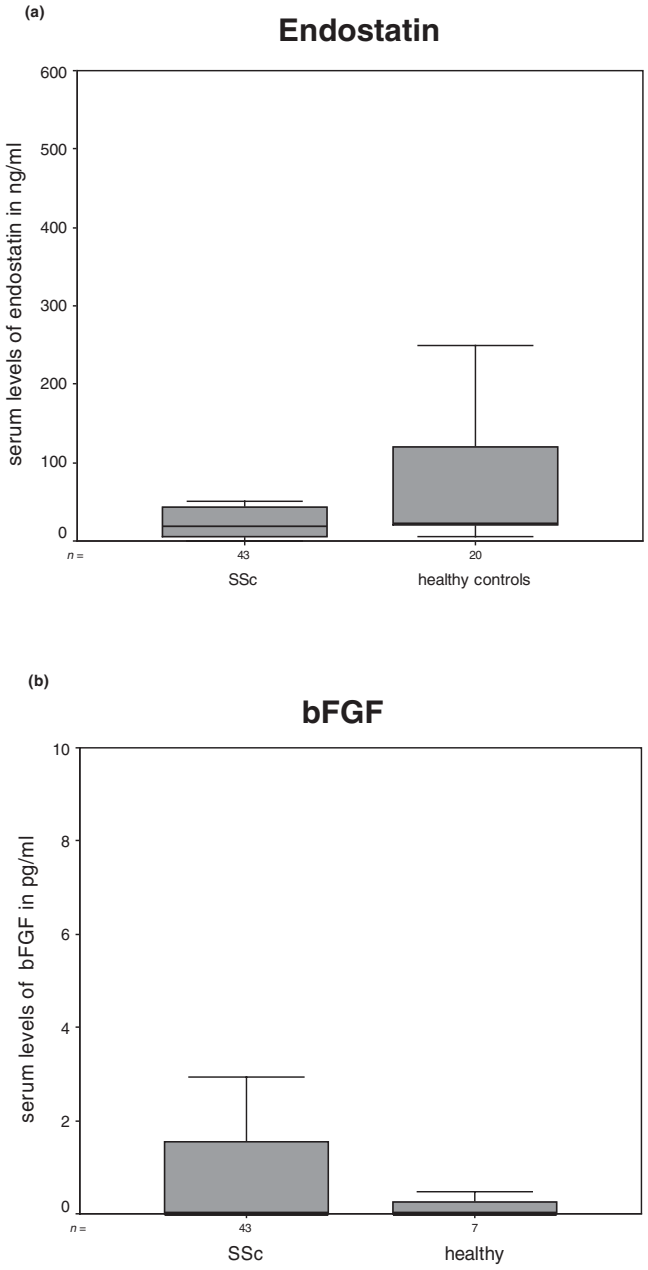
In contrast to VEGF, median values of endostatin were not increased in SSc patients (18.0 ng/ml; range, not detectable–750 ng/ml) compared with healthy controls (median, 22.5 ng/ml; range, 6–250 ng/ml) (Fig. 2a). Levels of endostatin were not different between patients with diffuse SSc (median, 18 ng/ml; range, not detectable–750 ng/ml) and patients with limited SSc (median, 20 ng/ml; range, 4–650 ng/ml; $P = 0.75$). Levels of bFGF were not detectable in the majority of patients with SSc ($n = 27/43$, 63%) and in healthy controls ($n = 5/7$, 71%) (Fig. 2b).

Figure 1



(a) Serum levels of vascular endothelial growth factor (VEGF) in patients with established systemic sclerosis (SSc) and in healthy controls. Data are shown as box plots, with upper and lower quartiles shaded. Highly significant differences were found for serum levels of VEGF compared with healthy controls. **(b)** Serum levels of VEGF analyzed according to the disease subset. Patients with diffuse SSc showed significant higher levels of VEGF than did patients with limited SSc. # $P < 0.05$.

Figure 2



Serum levels of **(a)** endostatin and **(b)** basic fibroblast growth factor (bFGF) in patients with established systemic sclerosis (SSc) and in healthy controls. Levels of endostatin and bFGF were not enhanced in the patients compared with healthy controls. Data are shown as box plots, with upper and lower quartiles shaded.

Disease duration and VEGF levels

To examine whether the upregulation of VEGF is a feature of the early stages of the disease or a secondary effect caused by regulatory mechanisms, serum samples were analyzed according to the disease duration.

Patients with pre-SSc (median, 487 pg/ml; range, 8–763 pg/ml) and patients with early SSc (median, 347 pg/ml; range, 93–1143 pg/ml) showed levels of VEGF that were in the range of those from patients with intermediate/late SSc (median, 424 pg/ml; range, 156–1151 pg/ml) (Fig. 3). In all groups including patients with pre-SSc, levels of VEGF were significantly higher than in healthy controls ($P < 0.001$). This indicates that the increased levels of VEGF are both early and persistent features of the disease. VEGF values were not significantly different between pre-SSc, early SSc and intermediate/late SSc ($P = 0.83$).

The group with pre-SSc patients was heterogeneous, in that 3/9 patients had levels of VEGF in the range of the normal controls, whereas 6/9 patients showed increased levels of VEGF. Patients from the pre-SSc group were again examined 1 year after inclusion into the study. Interestingly, at this followup, 4/6 pre-SSc patients with increased VEGF levels but none of the 3/9 pre-SSc patients with normal VEGF levels had developed definite SSc (numbers too low for statistical analysis).

Disease duration and endostatin and bFGF levels

In contrast to VEGF, levels of endostatin and bFGF were not significantly different between pre-SSc patients, SSc patients with different disease durations and healthy controls. Levels of bFGF were detectable in 4/9 patients with pre-SSc, in 2/9 patients with short disease duration and in 10/34 patients with longer disease duration.

Autoantibodies and VEGF levels

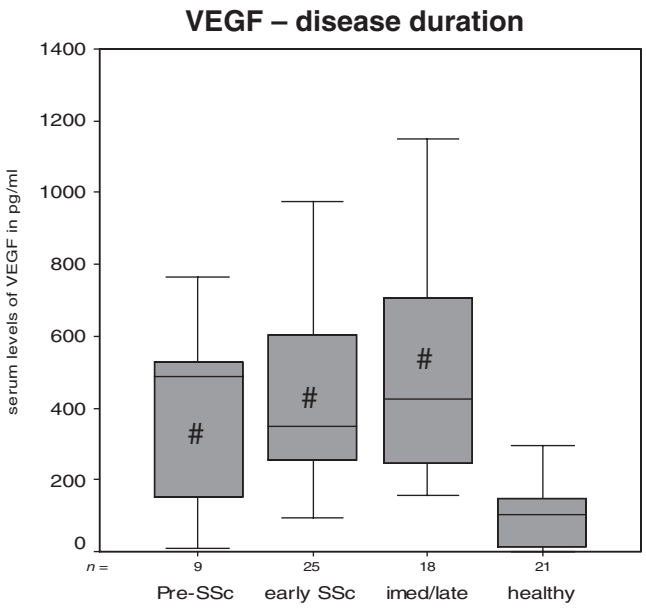
As illustrated in Fig. 4, the 13 patients with anti-Scl-70 autoantibodies showed significantly higher levels of VEGF (median, 706 pg/ml; range, 151–1151 pg/ml) than the 26 patients negative for anti-Scl-70 autoantibodies and positive for antinuclear antibodies (median, 339 pg/ml; range, 93–1013 pg/ml; $P \leq 0.04$), and they showed nonsignificantly higher levels than the four patients without detectable autoantibodies (median, 309 pg/ml; range, 135–612 pg/ml; $P = 0.11$).

No significant differences could be detected between patients with anticentromere antibodies (median, 339 pg/ml; range,143–1151 pg/ml), patients without anticentromere antibodies (median, 453 pg/ml; range, 93–1143 pg/ml) and patients without detectable autoantibodies ($P = 0.36$).

Autoantibodies and bFGF and endostatin levels

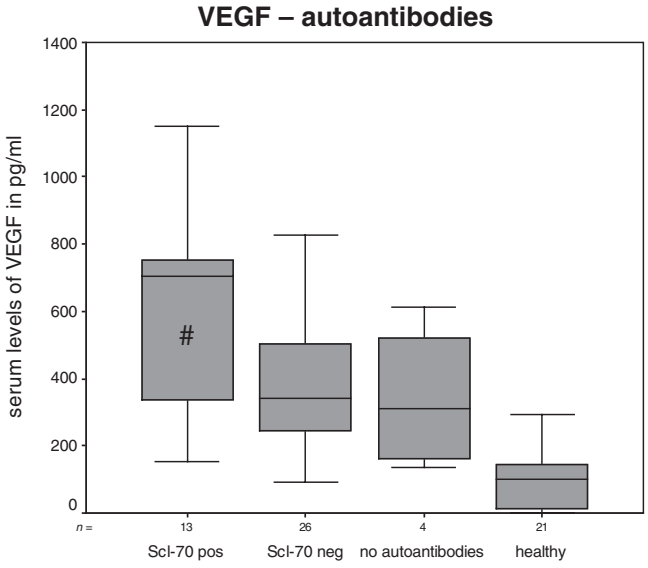
No association was found between levels of endostatin and the presence of anti-Scl-70 autoantibodies, anticentromere antibodies or antinuclear antibodies. Similarly, there was no association of bFGF with any of the autoantibodies.

Figure 3



Serum levels of vascular endothelial growth factor (VEGF) according to disease duration. The analysis included patients with pre-systemic sclerosis (pre-SSc) (autoantibodies, capillaroscopy changes and Raynaud's phenomenon, but not yet fulfilling American College of Rheumatology criteria), patients with early SSc (diffuse SSc < 3 years, limited SSc < 5 years) and patients with intermediate/late (imed/late) SSc (diffuse SSc ≥ 3 years, limited SSc ≥ 5 years). In all groups including patients with pre-SSc, VEGF levels were significantly increased compared with controls. No differences were found between patients with different disease duration. Data are shown as box plots, with upper and lower quartiles shaded. # $P < 0.05$.

Figure 4



Serum levels of vascular endothelial growth factor (VEGF) analyzed according to the presence of anti-Scl-70 autoantibodies. Patients with anti-topoisomerase I (Scl-70) autoantibodies (Scl-70 pos) showed significant higher levels of VEGF than patients without anti-Scl-70 autoantibodies (but positive for antinuclear antibodies) (Scl-70 neg) and higher levels than patients without detectable autoantibodies. Data are shown as box plots, with upper and lower quartiles shaded. # $P < 0.05$.

Capillaroscopy and VEGF levels

Serum levels of VEGF were increased in all capillaroscopy groups (early, active and late) compared with those in healthy controls. Patients with the early capillaroscopy pattern (median, 380 pg/ml; range, 195–754 pg/ml; $P < 0.001$), with the active pattern (median, 312 pg/ml; range, 93–1143 pg/ml; $P < 0.001$) and with the late pattern (median, 551 pg/ml; range, 156–1151 pg/ml; $P < 0.001$) all showed significantly higher levels of VEGF than the healthy control group. However, no significant differences in the levels of VEGF were found between the three capillaroscopy groups ($P = 0.32$).

Since the features of each capillaroscopy pattern are different but somewhat overlapping between the early, active and late groups, we also analyzed the levels of VEGF in relation to single capillaroscopy findings. Similar to the analyses with the capillaroscopy groups, no significant differences were found in the levels of VEGF between patients with a presence or an absence of avascular areas, giant capillaries, microhemorrhages and pericapillary edema.

Capillaroscopy and endostatin and bFGF levels

Serum levels of endostatin were not significantly different between the three capillaroscopy groups (early pattern: median, 85 ng/ml; range, 6–750 pg/ml; active pattern: median, 10 ng/ml; range, 0–500 ng/ml; late pattern: median, 19 ng/ml; range, 4–750 ng/ml) ($P = 0.15$).

Interestingly, the levels of endostatin showed an association with single microvascular findings as assessed by nailfold capillaroscopy (Table 2). Patients with giant capillaries showed significantly lower levels of endostatin than their counterparts without giant capillaries ($P \leq 0.02$). There were no differences in the levels of bFGF between the capillaroscopy groups and between the single capillaroscopy findings.

Fingertip ulcers and VEGF levels

Patients without fingertip ulcers showed significantly higher levels of VEGF (median, 413 pg/ml; range, 185–1151 pg/ml) than patients with the presence of fingertip ulcers (median, 280 pg/ml; range, 93–754 pg/ml; $P \leq 0.05$). This suggests that high levels of VEGF may be protective against the development of fingertip ulcers (Fig. 5a). Again, in both groups of patients, serum levels of VEGF were significantly higher than in healthy controls ($P < 0.001$ for both analyses).

Table 2

Association of endostatin levels and capillaroscopy findings

	Status	Median (ng/ml)	Range (ng/ml)	*P* value
Avascular areas	Present ($n = 14$)	20	4–750	0.13
	Absent ($n = 28$)	17	0–750	
Giant capillaries	Present ($n = 19$)	6	0–750	0.02
	Absent ($n = 23$)	20	4–750	
Hemorrhages	Present ($n = 15$)	18	0–750	0.19
	Absent ($n = 27$)	20	4–750	
Pericapillary edema	Present ($n = 37$)	18	0–750	0.18
	Absent ($n = 5$)	20	6–650	

Patients without giant capillaries showed significantly higher levels of endostatin than patients with giant capillaries. Similarly, there was a trend towards higher levels of endostatin in patients with avascular areas and in patients that did not have nailfold microhemorrhages and pericapillary edema.

When these parameters were analyzed according to the subset of the disease, even more pronounced differences were found between patients with diffuse SSc without fingertip ulcers ($n = 14$; median, 616 pg/ml; range, 281–1151 pg/ml) and patients with diffuse SSc with fingertip ulcers ($n = 9$; median, 280 pg/ml; range, 93–714 pg/ml; $P \leq 0.04$) (Fig. 5b). Patients with limited SSc showed less clear differences, which did not reach statistical significance, when analyzed according to the presence of fingertip ulcers (limited SSc without fingertip ulcers: $n = 13$; median, 332 pg/ml; range, 185–826 pg/ml; limited SSc with fingertip ulcers: $n = 7$; median, 187 pg/ml; range, 135–663 pg/ml) ($P = 0.36$).

Fingertip ulcers and endostatin and bFGF levels

There were no significant differences in the levels of endostatin between patients without fingertip ulcers (median, 15 ng/ml; range, 0–750 ng/ml) and those with fingertip ulcers (median, 20 ng/ml; range, 4–750 ng/ml; $P = 0.32$). Again, there was no association of bFGF levels with the presence of fingertip ulcers.

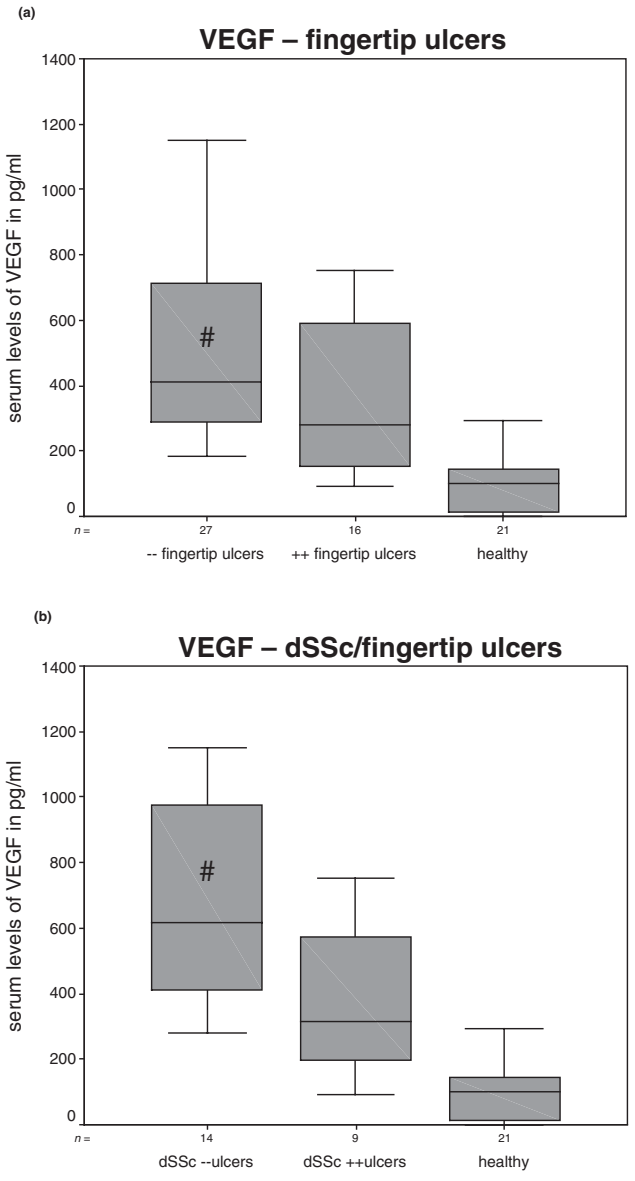
Levels of VEGF, endostatin and bFGF and other clinical parameters

No correlation of VEGF, endostatin and bFGF levels with skin score, carbon monoxide diffusion capacity and the presence of teleangiectasias and other skin ulcers was found.

Discussion

The process of angiogenesis appears to be largely impaired in SSc following the profound disarrangement of the microcirculation. The damage of the vessels evolves progressively from early to late stages and is characterized by different morphological aspects.

Figure 5



Serum levels of vascular endothelial growth factor (VEGF) in systemic sclerosis (SSc) patients with and without fingertip ulcers. Compared with healthy controls, serum levels of VEGF were increased in patients with fingertip ulcers. In patients without fingertip ulcers, however, levels of VEGF were even more enhanced, indicating that VEGF might be protective against the development of fingertip ulcers if its serum concentration exceeds a certain threshold level. Data are shown as box plots, with upper and lower quartiles shaded. # $P < 0.05$.

The present study shows clearly that circulating levels of VEGF are increased in SSc, in the range of those reported for patients with breast cancer, lung cancer and other malignancies [22,23]. Numerous studies have shown that VEGF plays a crucial role in the formation of tumor vessels, which are in turn critical for nourishment and growth of these tumors [24,25]. As an example, treatment

with anti-VEGF antibodies of nude mice injected with different tumor cell lines leads to a nearly complete suppression of tumor-associated angiogenesis and to a rapid inhibition of tumor growth [26].

Median serum levels of VEGF in patients with rheumatoid arthritis are also comparable with those found for SSc patients in the present study, and blockade of VEGF reduced the disease severity in murine collagen-induced arthritis [27,28]. However, while VEGF plays a dominant role in the formation of new vessels in malignancies and inflammatory disorders such as rheumatoid arthritis, it is unclear whether similar levels of VEGF can lead to sufficient neoangiogenesis in SSc.

Considering that only a small increase in VEGF protein results in efficacious new vessel formation in a variety of animal models and that angiogenesis is regulated by a balance of angiogenesis inducers and inhibitors, possible explanations for an insufficient angiogenesis in SSc include a blockade of the biologic effects of VEGF by one or more angiostatic factors [25,29]. Although other reasons such as signaling defects or lack of the VEGF receptors flt and flk cannot be excluded with the present study, this hypothesis is strongly supported by the fact that patients without fingertip ulcers showed highly elevated serum concentrations of VEGF. Serum levels of VEGF were also found to be elevated in patients with fingertip ulcers, but these levels may not have been sufficiently high to override the effects of angiostatic factors. Notably, the association between fingertip ulcers and VEGF serum concentrations was significant only in patients with diffuse SSc, indicating a potential discrepancy between the pathways leading to fingertip ulcers in the two subsets of the disease.

A decrease of angiogenic factors might be expected in ischemic diseases such as SSc. Paradoxically, our study shows an increase of VEGF in the serum of patients with SSc compared with healthy controls. The triggers as well as the source of VEGF in serum samples of SSc patients remain to be defined. Platelets have been shown to release VEGF after stimulation [30]. Hypoxia increases the synthesis of VEGF in a variety of cell types via an accumulation of the transcription factor hypoxia inducible factor 1 [31]. In addition, a variety of cytokines (e.g. interleukin-1, transforming growth factor beta and platelet-derived growth factor) known to be upregulated in SSc induce the synthesis of VEGF [32–34].

The present data suggest that, although levels of VEGF are already elevated, a further increase of VEGF might be a therapeutic option for SSc patients with fingertip ulcers. In fact, encouraging animal studies led to clinical trials using recombinant VEGF or gene therapy in patients with different ischemic diseases. In a phase I study with recombinant $VEGF_{165}$ in patients with coronary ischemia, the therapy was safely tolerated and resulted in improved perfusion and collateralization in a subset of patients [25]. Similarly, intramuscular gene transfer of naked plasmid DNA encoding for $VEGF_{165}$ ($phVEGF_{165}$) in patients with critical limb ischemia showed an improvement in several hemodynamic and angiographic parameters without major complications [35].

Whereas VEGF might on one hand have favorable effects in the prevention of fingertip ulcers, the present study provides evidence that it might, on the other hand, contribute to the progression and severity of SSc. Tissue edema of the distal extremities in particular, resulting in 'puffy digits', is a typical feature of the early 'edematous' phase of SSc, and has been proposed as a potential trigger for fibroblast activation [3]. VEGF was initially named vascular permeability factor because of its ability to promote the extravasation of plasma proteins from blood vessels [36]. Prominent edema of the lower extremity was found in more than 30% of patients with critical limb ischemia after gene transfer of $phVEGF_{165}$ [37]. The hypothesis that VEGF might have dual functions in the pathogenesis of SSc, with positive effects on the vascular system but with negative effects on the development of fibrosis, has to be tested in functional studies (e.g. by application of VEGF in animal models of SSc and by careful assessment of both vascular and fibrotic parameters).

The increase of VEGF in patients with the earliest disease stages found in the present study argues for an important role of VEGF in the pathogenesis of early vascular, and possibly fibrotic, changes. Along this line, levels of VEGF were increased in patients with anti-topoisomerase antibodies and diffuse SSc, which are associated with a more rapid and severe disease course [38]. These results are consistent with findings from Kikuchi *et al*., who showed a correlation of VEGF with the frequency of lung fibrosis and reduced vital capacity in patients with SSc [39]. An important observation of the present study is the development of cutaneous involvement in pre-SSc patients with increased levels of VEGF. Prospective studies with larger patient numbers are needed to confirm this finding. In addition, the classification of patients with Raynaud's phenomenon plus nailfold capillary changes and disease-specific autoantibodies as 'pre-SSc' patients is controversial and might favor patients with limited SSc.

According to the hypothesis that the angiogenic effects of VEGF are inhibited by a concomitant increase of angiostatic factors, another strategy for the treatment of ischemic symptoms in SSc is the inhibition of angiostatic factors rather than a further increase of VEGF. Angiostatic factors are often cleaved enzymatically from extracellular matrix proteins [40]. Among these extracellular matrix-derived angiostatic growth factors, endostatin has been

characterized as a potent inhibitor of VEGF-induced angiogenesis [41].

The inverse association of endostatin with giant capillaries and microhemorrhages and of the higher levels of endostatin in patients with avascular areas argue for a role of endostatin in the pathogenesis of microvascular abnormalities in SSc. For example, Hebbar *et al*. found a correlation of endostatin with cutaneous ulcers [42]. However, in contrast to their findings, endostatin was elevated only in a small number of SSc patients, and no association was found with any other clinical parameter.

The reasons for these differences are not clear. Enzyme immunoassays for the determination of endostatin were purchased from the same manufacturer (Cytimmune) and serum samples were processed in a similar way, thereby making methodological differences unlikely. Interestingly, healthy controls showed similar levels of endostatin, while patients with SSc had much lower levels in our study. Possible explanations therefore include clinical differences in the study populations. In fact, patients in our study were older and more often had diffuse SSc as well as anti-Scl-70 autoantibodies than those in the study from Hebbar *et al*. [42]. Whether enhanced levels of endostatin might be specific for certain subgroups of SSc remains to be examined in further studies. To date, these data suggest that while endostatin may contribute to microvascular changes in some patients, the lack of a sufficiently functioning microvascular network in SSc is more probably mediated by a concerted action of several angiostatic factors that remain to be identified.

Conclusion

The present study provides evidence that VEGF might have protective effects against the development of fingertip ulcers, and thereby suggests that an increase of VEGF might be a therapeutic option for patients with SSc. VEGF might also contribute to the progression of the disease, however, and possible drawbacks of a therapy with a single angiogenic growth factor have to be carefully considered. The present study indicates further that the biologic effects of VEGF are counteracted by a concerted action of several angiostatic factors rather than by endostatin alone.

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Correspondence

Steffen Gay, MD, Center of Experimental Rheumatology, Department of Rheumatology, University Hospital Zurich, CH-8091 Zurich, Switzerland. Tel: +41 1255 2962; fax: +41 1255 4170; e-mail: Steffen.Gay@ruz.usz.ch