Uncontrolled Expression of Vascular Endothelial Growth Factor and Its Receptors Leads to Insufficient Skin Angiogenesis in Patients With Systemic Sclerosis


Abstract—Systemic sclerosis (SSc) skin lesions are characterized by disturbed vessel morphology with enlarged capillaries and an overall reduction in capillary density, suggesting a deregulated, insufficient angiogenic response. It has been postulated that this phenomenon is due to reduced expression of the potent angiogenic factor vascular endothelial growth factor (VEGF). In contrast to this hypothesis, we demonstrate that the expression of both VEGF and its receptors VEGFR-1 and VEGFR-2 is dramatically upregulated in skin specimens of SSc patients throughout different disease stages. Interestingly, upregulation of VEGF was not mediated by hypoxia-inducible transcription factor-1 (HIF-1) as indicated by only a weak expression of the oxygen-sensitive α-subunit of HIF-1 in the skin of SSc patients. This was unexpected on measuring low Po2 values in the SSc skin by using a polarographic oxygen microelectrode system. Considering our observation that PDGF and IL-1β costimulated VEGF expression, we propose that chronic and uncontrolled VEGF upregulation that is mediated by an orchestrated expression of cytokines rather than VEGF downregulation is the cause of the disturbed vessel morphology in the skin of SSc patients. Consequently, for therapeutic approaches aiming to improve tissue perfusion in these patients, a controlled expression and timely termination of VEGF signaling appears to be crucial for success of proangiogenic therapies. (Circ Res. 2004;95:109-116.)

Key Words: angiogenesis ■ vascular endothelial growth factor ■ hypoxia-inducible transcription factor-1

Systemic sclerosis (SSc) is a multiorgan disease characterized by widespread fibrosis, activation of immune cells, production of autoantibodies, and injury to vascular as well as microvascular structures. The earliest clinical symptoms of SSc relate to disturbances in the peripheral vascular system. Nailfold capillaroscopy shows a variety of morphological changes including enlarged capillaries, bushy capillary formations, microhemorrhages, and a variable loss of capillaries with or without avascular areas. These phenomena are often accompanied by increased markers of endothelial cell injury and endothelial cell activation.

Despite the reduced capillary density, there is paradoxically no sufficient angiogenic response in the skin of patients with SSc. Tissue ischemia leads usually to the expression of angiogenic growth factors, which then initiate angiogenic sprouting by inducing vasodilatation, proliferation, and migration of endothelial cells and stabilization of the lumina to form new vessels.

Among the angiogenic growth factors, vascular endothelial growth factor (VEGF) has been identified as a key mediator of angiogenesis. VEGF induces differentiation, proliferation, and migration of endothelial cells that contribute to the formation of vessels through both angiogenesis and vascular remodeling. VEGF exerts its biological functions by binding to the tyrosine kinase receptors VEGFR1 (Flt-1) and VEGFR2 (Flk-1). Deletion of VEGF as well as of its receptors lead to embryonic lethality due to vascular defects, further highlighting the complex and multiple functions of the VEGF/VEGF receptor axis for angiogenic processes (see review). The expression of VEGF is tightly controlled and stimulated under hypoxic conditions through binding of the transcription factor hypoxia-inducible transcription factor-1 (HIF-1), a heterodimeric transcription factor consisting of the oxygen-regulated α-subunit and the constitutively expressed β-subunit, to a 47-bp sequence located 985 to 939 bp upstream of the transcription initiation site.

Considering the lack of a sufficient angiogenesis despite the reduction in the capillary density, we hypothesized that the expression of the potent proangiogenic growth factor...
VEGF might be downregulated in patients with SSc. In the present study, we therefore analyzed the expression and regulation of VEGF and its receptors in skin specimens from SSc patients. In addition, we addressed the impact of HIF-1α on VEGF expression in SSc patients.

Materials and Methods
For an expanded Materials and Methods, see the online data supplement available at http://circres.ahajournals.org. Characteristics of SSc patients are shown in online Table 1.

Patients and Skin Biopsies
Skin biopsies were obtained from 15 patients who met the American College of Rheumatology criteria for SSc. In all patients, biopsies were taken from clinically involved skin. In a subset of patients (n=7), biopsies were also taken from clinically noninvolved skin as assessed by skin scoring of an experienced examiner. Controls (n=6) consisted of biopsies from healthy volunteers. Experiments were approved by local ethics review committees, and written informed consent was obtained from all patients.

In Situ Hybridization
Plasmids containing the 517 bp human VEGF121 cDNA were kindly provided by H.A. Weich (Max-Planck-Institute, Bad Nauheim, Germany) and used as a template to design VEGF-A–specific riboprobes. Preparation of VEGF-A–specific riboprobes and non-radioactive in situ hybridization was performed as described elsewhere. Hybridized probes were visualized with nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Measurement of Skin Oxygenation
Skin oxygenation was measured intradermally in 13 SSc patients and 5 healthy controls at the dorsal aspect of the forearm (midway between wrist and elbow) using a PO2 histograph (Eppendorf, Germany). Healthy controls were age- and sex-matched with the SSc patients.

Cell Culture and Hypoxic Induction
Fibroblast cultures were obtained from skin biopsies of affected skin of additional patients. Control fibroblast cultures (n=5) were obtained from healthy subjects. For exposure to hypoxia, fibroblasts were grown to 50% to 80% confluence and transferred into a hypoxic incubator (Forma Scientific) containing 1% O2 v/v (hypoxia, oxygen tension 7 mm Hg) or 20% O2 v/v (normoxia, oxygen tension 140 mm Hg).

Western Blot Analysis and Immunofluorescence
Cultured cells were removed from the hypoxic incubator and rinsed quickly with ice-cold PBS. After cell lysis, extraction of nuclear proteins, and transfer on nitrocellulose membranes, HIF-1α protein was detected using monoclonal mouse anti–HIF-1α mgc3 antibodies. Similarly, for immunofluorescence on cultured cells, monoclonal mouse anti–HIF-1α mgc3 antibodies were used. Bound antibodies were visualized using FITC conjugated goat anti-mouse antibodies.

Real-Time PCR
In all stimulation experiments, total RNA was isolated using the TRIzol LS reagent (Gibco/Life Technologies) according to the manufacturer’s protocol. Reverse transcription and TaqMan real-time PCR specific for VEGF-A was performed as described recently. 11 18S was used as an endogenous control. Results were quantified with the threshold cycle (Ct) and the comparative Ct method. All experiments as well as all real-time PCR measurements were performed in duplicates.

Immunohistochemistry
HIF-1α protein was detected as described recently using monoclonal mouse anti–HIF-1α antibodies (Novus). For Flk-1/VEGFR2, monoclonal mouse antibodies were used. Flt-1/VEGFR1 protein was detected using polyclonal rabbit antibodies (both from Santa Cruz Biotechnology). For double labeling experiments, monoclonal mouse anti–CD68–antibodies (clone PG-M1, Dako) were used. Serial sections of in situ hybridization and VEGFR immunohistochemistry were stained with polyclonal rabbit anti-von Willebrand-Factor antibodies (A 0082, Dako).

Stimulation With Platelet-Derived Growth Factor and Interleukin-1β
Cultured dermal fibroblasts were grown to confluence in 24-well plates and stimulated with recombinant platelet-derived growth factor-BB (PDGF-BB) at 10 and 40 ng/mL and with recombinant interleukin-1β (IL-1β) at 1 pg/mL, 10 pg/mL, and 100 pg/mL. Recombinant proteins were purchased from R&D Systems (Abingdon, UK). Costimulation experiments were performed with 40 ng/mL PDGF-BB and 100 pg/mL IL-1β.

Statistics
Data are shown as median with range. The Mann-Whitney test was used for statistical analysis. A value of P<0.05 was considered statistical significant.

Results
VEGF mRNA Is Upregulated in Skin Biopsies From Patients With SSc
Consistent with recent findings, a constitutive expression of VEGF could be detected in the epidermis of healthy controls (Figure 1A). Whereas in some healthy controls the expression of VEGF was limited to suprabasal layers (Figure 1A), others showed a more scattered pattern of expression throughout the epidermis. As assessed by scoring in randomly chosen high-power fields, the mean percentage of keratinocytes expressing VEGF was 14% (range 0% to 30%). None of the healthy controls showed an expression of VEGF in dermal cells.

In contrast and despite the lack of sufficient angiogenesis in the SSc skin, an upregulation of VEGF was found in affected skin biopsies from patients with SSc (Figure 1B). The mean percentage of keratinocytes expressing VEGF was increased to 50% (range 0% to 100%, P<0.05 compared with healthy controls). In addition to the enhanced expression in the epidermis, an enhanced expression of VEGF could be detected in the dermis of 13/15 patients. VEGF was expressed by a variety of cell types including fibroblasts and endothelial cells and inflammatory cells (Figure 1C and 1D). For double labeling experiments, see online data supplement. Interestingly, biopsy specimens taken from clinically noninvolved skin from the same patients (n=7) showed the same expression pattern of VEGF in each patient as observed for involved skin biopsies.

Hypoxia Is Present in Skin Lesions of SSc Patients
Given the strong upregulation of VEGF in skin biopsies of SSc patients, we next searched for possible stimulators of VEGF in the SSc skin. Using a PO2 histogram to measure tissue oxygen levels, SSc patients with nonfibrotic skin at the site of measurement (forearm) did not show different levels of intracutaneous PO2 values when compared with healthy
controls. SSc patients with involved (fibrotic) skin at the site of measurement, however, had strikingly lower levels of P O 2 than healthy controls and SSc patients without fibrotic changes at the forearm (P<0.05; Table). The lower levels of oxygen in the involved skin were not caused by systemic parameters, because no differences between the groups were found for arterial oxygen saturation, hemoglobin content, blood pressure, and heart rate (data not shown).

Characterization of the HIF-1α Induction by Hypoxia in Dermal Fibroblasts

Because the transcription factor HIF-1 is the key molecule regulating molecular responses to hypoxia, we next aimed to characterize the expression of its oxygen-dependent α-subunit (HIF-1α) in cultured dermal fibroblasts under hypoxic conditions. Note that the oxygen concentration used in these experiments (1% O2) is equivalent to a P O 2 value of 7 mm Hg, which is close to the 10% percentile measured in fibrotic skin areas of SSc patients. An expression of HIF-1α was found by Western blotting after 6 hours of hypoxic exposure, whereas no signal could be detected after shorter exposure periods as well as in normoxic fibroblasts (Figure 2A). The accumulation of HIF-1α protein was maintained during prolonged exposure to hypoxia for 12, 24, and 48 hours at a similar expression level (Figure 2B). Interestingly, when these experiments were performed under low-serum conditions, the accumulation of HIF-1α under hypoxic conditions was considerably lower than in conditions using 10% fetal calf serum. This effect was seen in both SSc and normal fibroblasts (Figure 2B).

The HIF-1α protein needs to be translocated from the cytosol into the nucleus to induce molecular responses to hypoxia via binding to its HIF-binding site present in the flanking regions of HIF-target genes. As analyzed by indirect immunofluorescence, HIF-1α was almost completely located in the nucleus in dermal fibroblasts as early as 6 hours after hypoxic exposure (Figure 2C). No expression of HIF-1α was found by immunofluorescence in cells cultured under normoxic conditions. Taken together, these data indicate that HIF-1α can readily be activated under the given parameters in cells derived from SSc patients.

Effects of Hypoxia on VEGF Levels and Expression of HIF-1α Protein in SSc Skin Biopsies

We analyzed next whether the observed upregulation of HIF-1α by hypoxia is accompanied by an induction of VEGF in cultured dermal fibroblasts from SSc patients. As expected, hypoxic exposure for 24, 48, and 96 hours led to increased levels of VEGF mRNA in all cultures compared with fibroblasts kept under normoxic conditions (Figure 3). This induction of VEGF mRNA was of statistical significance at all time points (P<0.05) in both SSc and normal fibroblasts.

The experiments mentioned earlier suggested strongly that the abundant expression of VEGF in the SSc skin is mediated by HIF-1α that per se might have been activated by low P O 2 levels in fibrotic skin areas of SSc patients. To address this hypothesis, we analyzed the expression of HIF-1α protein by immunohistochemistry in skin specimens from SSc patients and healthy controls and compared them with the expression of VEGF in the same subjects.
In skin specimens from healthy controls, nuclear signals for HIF-1α were detected in the epidermis with a moderate to high expression in all subjects (Figure 4A). (For detailed semiquantitative analysis, see online Table 2.) VEGF showed a similar pattern of expression in serial sections from the same healthy controls (Figure 4C). This coexpression of HIF-1α and VEGF in healthy subjects indicates that the constitutive expression of VEGF in the epidermis might in fact be driven by HIF-1α. In parallel with the results for VEGF, no expression of HIF-1α could be detected in the dermis of healthy controls.

### Intracutaneous Levels of PO2 From Healthy Controls and SSc Patients

<table>
<thead>
<tr>
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<th>Mean PO2, mm Hg</th>
<th>Median PO2, mm Hg</th>
<th>10% Percentile PO2, mm Hg</th>
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<tr>
<td>Healthy controls</td>
<td>33.6 ± 4.1</td>
<td>32.9 ± 4.2</td>
<td>21.1 ± 2.7</td>
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<tr>
<td>SSc nonfibrotic</td>
<td>37.9 ± 8.6</td>
<td>38.3 ± 9.8</td>
<td>22.3 ± 5.6</td>
</tr>
<tr>
<td>SSc fibrotic</td>
<td>23.7 ± 2.1*</td>
<td>21.7 ± 4.0*</td>
<td>8.9 ± 3.2*</td>
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Levels of PO2 were measured at the forearm by 400 single PO2 measurements in each subject. Mean, median, and 10% percentile PO2 values were calculated for each subject. SSc patients with fibrotic skin at the site of measurements showed significantly lower levels of PO2 than healthy controls and SSc patients without fibrotic changes at the site of measurement in all parameters analyzed. *P<0.05.

Figure 2. Representative Western blots for HIF-1α using nuclear protein extracts from cultured cells. HELA cells were used as positive controls. Anti–Sp-1 antibodies were used to control for equal loading of proteins. A, Time-dependent induction of HIF-1α in SSc fibroblasts under hypoxic conditions. Expression of HIF-1α could be detected after 6 hours, whereas no signal was found after shorter exposure times to hypoxia and in normoxic controls. B, Effects of long-term hypoxia (48 hours) and of low-growth factor conditions on HIF-1α expression. Expression of HIF-1α in dermal fibroblasts was maintained after prolonged exposure to hypoxia at a similar level of expression. Low growth factor conditions (1% FCS) reduced the expression of HIF-1α. C, Immunofluorescence with anti–HIF-1α antibodies on SSc dermal fibroblasts after 6 hours of hypoxic incubation (1% O2) showing almost complete translocation into the nucleus.
Surprisingly, the expression of HIF-1α protein in skin specimens from SSc patients was lower than in those from healthy subjects. In the epidermis, we found weak signals for HIF-1α in a limited number of keratinocytes, which contrasted with the abundant epidermal expression of VEGF in these biopsies (Figure 4B and 4D). In addition, the expression pattern of HIF-1α did not correlate with the expression pattern of VEGF. Consistent with this observation, no HIF-1α protein was detected in dermal cells of SSc patients, despite the strong upregulation of VEGF in the dermis. Taken together, these data suggest that HIF-1α-independent mechanisms contribute to the upregulation of VEGF in the SSc skin.

Expression of VEGF in Dermal Fibroblasts

To compare the normoxic constitutive synthesis of VEGF in dermal fibroblasts from SSc patients and healthy controls, cells were grown to confluence and quantified for VEGF expression by real-time PCR. VEGF mRNA could be detected in all healthy and SSc fibroblast cultures with some variability between individual fibroblasts in both groups. In contrast to the observation in skin biopsies, no significant difference were detected between SSc and normal fibroblasts (median ΔCt normal: 8.2, range 6.2 to 8.6; median ΔCt SSc: 8.4, range 6.4 to 8.7). Similarly, under low growth factor conditions (1% FCS), no differences were found between fibroblasts derived from healthy controls and SSc patients (median ΔCt normal: 18.0, range 17.2 to 18.7; median ΔCt SSc: 17.8, range 17.3 to 19.1).

Cytokines Induce VEGF in Dermal Fibroblasts

Because HIF-1α does not appear to be a major mediator of VEGF upregulation in the SSc skin, we focused on PDGF as well as IL-1, both factors being implicated as key molecules in the pathogenesis of SSc in a number of previous studies.15–18 When fibroblasts were treated with recombinant PDGF, there was a dose-dependent increase of VEGF mRNA in both SSc and normal fibroblasts (10 ng/mL: normal, 1.6±0.4-fold increase; SSc, 1.6±0.3-fold increase; 40 ng/mL: normal, 3.0±0.8 fold increase; SSc, 1.8±0.5-fold increase; P<0.05 compared with nonstimulated controls).

Treatment with recombinant IL-1β resulted also in a dose-dependent increase of VEGF mRNA in all fibroblast cultures. Inductions were small but significant, reaching a 2.4±0.6-fold increase of VEGF mRNA in SSc fibroblasts.
and 1.9±0.2-fold increase in normal fibroblasts at concentrations of 100 pg/mL (P<0.05 compared with nonstimulated controls).

In vivo, SSc dermal fibroblasts are exposed to a variety of cytokines that are overexpressed in the SSc skin including PDGF and IL-1β. To mimic this situation, cultured cells were costimulated with recombinant PDGF and IL-1β and analyzed for VEGF mRNA levels. Whereas the stimulation with either PDGF or IL-1β resulted in a rather small increase of VEGF, the costimulation with 100 pg/mL IL-1β and 40 ng/mL PDGF showed additive effects with a strong increase of VEGF (Figure 5). The induction of VEGF raised to 4.7±0.5-fold increase in SSc fibroblasts compared with a 2.0±0.4 fold increase with IL-1β alone and a 2.3±0.2 fold increase with PDGF alone. These data indicate that the strong upregulation of VEGF in the SSc skin might be a net effect of different cytokines rather than an effect of a single factor.

Upregulation of VEGF Receptors in Systemic Sclerosis

Possible explanations for the lack of a sufficient angiogenesis in the SSc skin despite the upregulation of VEGF include a reduced expression of VEGF receptors (VEGFRs) on endothelial cells. To address this possibility, immunohistochemistry with antibodies against Flt-1/VEGFR1 and Flk-1/VEGFR2 was performed on skin sections of SSc patients and healthy controls. Expression of Flt-1/VEGFR1 was found in all biopsies from SSc patients (Figure 6A). VEGFR1 has been described as an endothelial specific molecule. Consistent with these reports, the expression of VEGFR1 was limited to endothelial cells of smaller vessels in the SSc skin (see also online Figure 3). In contrast to the significant expression in the SSc skin, expression of VEGFR1 could not be detected in 5/6 biopsies from healthy controls (Figure 6B), whereas one healthy control showed a weak expression on endothelial cells.

Similar to VEGFR1, the expression of Flk-1/VEGFR2 was found to be upregulated in SSc skin biopsies (Figure 6C). In agreement with the in situ hybridization for VEGF, VEGFR2 was found expressed in 13/14 patients. Again, the expression of VEGFR2 was preferentially detected on endothelial cells (see also online Figure 4). In general, the number of endothelial cells expressing VEGFR2 and the intensity of staining was higher than for VEGFR1. No other dermal structures than endothelial cells expressed VEGFR2, whereas some positive signals were observed in the epidermis from both SSc patients and healthy controls (Figure 6C and 6D). Interestingly, whereas in contrast to normal skin, both VEGF receptors were expressed also in biopsies from noninvolved (nonfibrotic) skin of SSc patients, the percentage of endothelial cells expressing both receptors was lower than in biopsies of involved (fibrotic) skin of the same patients (see online Tables 3 and 4 for detailed semiquantitative analysis of VEGFR1 and VEGFR2 immunohistochemistry).
Discussion

In the present study, we addressed the hypothesis that the lack of sufficient angiogenesis in SSC might be explained by a downregulation of the potent angiogenic factor VEGF, which is a key molecule in several steps of angiogenesis. However, unexpectedly, skin specimens of patients with SSC showed a strong upregulation of VEGF by in situ hybridization compared with healthy controls. Apart from VEGF, also its receptors VEGF-R1 and VEGF-R were found to be upregulated on endothelial cells of SSC patients in vivo. In addition, we could show recently that VEGF protein was significantly increased in blood samples from patients with SSC, reaching levels observed in patients with numerous malignant diseases. Taken together, these data strongly imply an activation of the VEGF/VEGF-receptor axis in patients with SSC.

Angiogenesis is regulated by a tightly controlled balance of angiogenic and angiostatic factors. Thus, an attractive hypothesis for the insufficient angiogenesis in SSC despite upregulated increased levels of VEGF could be an upregulation of angiostatic factors that is even higher than those of VEGF and thereby outweighs its proangiogenic effects. However, data on angiostatic factors in SSC are inconsistent. For instance, the collagen type XVIII breakdown product endostatin has been described as significantly increased or not different from healthy controls. Recently, Macko et al. found elevated levels of platelet factor-4 and thrombospondin-1 in plasma samples from patients with SSC.

Of particular interest regarding the role of VEGF in the pathogenesis of SSC is the increasing experimental evidence about the diverse biological actions of VEGF depending on the period and level of expression. Using pTET-VEGF transgenic mice, in which the expression of VEGF can be conditionally switched off in an organ-dependent manner by feeding tetracycline, Dor et al. showed that overexpression of VEGF induces the formation of new functional vessels in adult organs. These vessels were mature as reflected by the presence of smooth muscle cells and resulted in an improved organ perfusion. However, most interestingly, prolonged exposure to VEGF without subsequently switching off its expression resulted in the formation of irregularly shaped sac-like vessels with reduced blood flow, very much reminiscent of the disturbed vessel morphology with megacapillaries seen in SSC patients. In fact, the timely downregulation of VEGF rather than the induction of angiostatic factors appears to be the major mechanism preventing the formation of chaotic vessel morphology. This is further evidenced by the association of chronic overexpression of VEGF with glomeruloid and hemangioma-like vessels in other experimental settings. Thus, although short-time upregulation of VEGF is a strong inducer of angiogenesis, chronic and uncontrolled overexpression of VEGF leads to chaotic vessel morphology with reduced blood flow in the newly formed vessels.

In the present study, overexpression of VEGF in the skin specimens was detected independent of the disease duration in both patients with early as well as late disease. In addition, recent detailed analysis of blood levels in SSC revealed that VEGF was significantly upregulated in patients with different disease durations including patients with very early stages that not yet fulfilled ACR criteria, but did so during a 1-year follow-up. Furthermore, cytokines known to induce the synthesis of VEGF are overexpressed in SSC throughout the disease course. In addition to PDGF and IL-1, this also includes TGFβ, which is considered a key factor in the development of fibrosis in SSC. Taken together, these data indicate that a chronic and uncontrolled overexpression of VEGF does occur in SSC and might significantly contribute to the chaotic capillary morphology seen in these patients. The uncontrolled overexpression of VEGF does already exist in earliest disease stages in parallel with the morphological capillary changes observed by capillary microscopy. The chronic expression appears to be driven by key cytokines in the pathogenesis of SSC including PDGF and IL-1 throughout different disease stages.

We also addressed other possible stimulators of VEGF in the SSC skin. Surprisingly, despite the clear presence of hypoxia in the involved skin of patients with SSC and despite the nuclear accumulation of HIF-1α in cells derived from SSC patients under hypoxic conditions in vitro, the expression of HIF-1α protein was lower in skin specimens from SSC patients than in healthy controls. Although in vitro the stabilization of HIF-1α protein is instantaneous and sustained for up to 48 hours in dermal fibroblasts, the response in vivo might be substantially different. Consistent with our findings, it has been reported that exposure of mice to 6% hypoxia leads to maximum levels of HIF-1α protein in the brain after 4 to 5 hours, but declines afterward, reaching basal normoxic levels after 9 to 12 hours. Similar results were obtained for the kidney and the liver. Considering the correlation of low oxygen levels with fibrotic structural changes in the skin of patients with SSC in the present study, hypoxia in SSC patients appears to be chronic rather than caused by acute situations such as vasoconstriction in the setting of Raynaud’s phenomenon. Whether the response of HIF-1α to chronic hypoxia is similar in the skin as reported for other organs has not yet been addressed. It has to be stressed, however, that our findings do not rule out that pathways induced by hypoxia independent from HIF-1α (e.g., via induction of other members of the HIF family) significantly contribute to the pathogenesis of SSC. This is further supported by recent data showing that hypoxia directly contributes to the accumulation of extracellular matrix both by HIF-1α-dependent as well as HIF-1α-independent mechanisms.

In summary, we have shown that, despite the lack of a sufficient angiogenesis in SSC, the proangiogenic factor VEGF together with its receptors VEGF-R1 and VEGF-R are upregulated in skin specimens from SSC patients. Although involved skin is characterized by strongly reduced levels of oxygen, hypoxia-induced HIF-1α does not appear to be the major mediator of VEGF induction in SSC. Instead, the chronic and uncontrolled overexpression of VEGF is based on a net effect of cytokines such as IL-1 and PDGF. SSC might therefore serve as a disease model for the effects of a chronic and unterminated upregulation of VEGF with the formation of irregularly shaped sac-like megacapillaries. Consequently, for therapeutic approaches aiming to improve
tissue perfusion in these patients, a controlled expression and timely termination of VEGF signaling appears to be crucial for success of proangiogenic therapies.

Acknowledgments

The study was supported by the Deutsche Akademie der Naturforscher Leopoldina (grant BMBF-LPD 9901/8-7), the Braun Foundation, and grant 560031 of the University of Zurich. The authors thank Ferenc Pataky and Maria Comazzi for excellent technical assistance.

References


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Circ Res. 2004;95:109-116; originally published online June 3, 2004; doi: 10.1161/01.RES.0000134644.89917.96

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Materials and Methods

Patients and skin biopsies
Skin biopsies were obtained from 15 patients who met the American College of Rheumatology criteria for SSc \(^1\). In all patients, biopsies were taken from clinically involved skin. In a subset of patients (n = 7), biopsies were also taken from clinically non-involved skin as assessed by skin scoring of an experienced examiner. Tissue samples of 6/15 patients were fixed in 4% buffered formalin for 6 hours, transferred to 50% ethanol and embedded in paraffin \(^2\). Skin biopsies of 9/15 patients with SSc were snap frozen in O.C.T. Tissue Tek embedding medium (Miles, Elkhart, IN) and stored at -80\(^\circ\) C. Controls (n = 6) consisted of biopsies from healthy volunteers. Experiments were approved by local ethics review committees, and written informed consent was obtained from all patients.

Cell culture and hypoxic induction
Fibroblast cultures were obtained from skin biopsies of affected skin of additional patients. Control fibroblast cultures (n = 5) were obtained from healthy subjects. After enzymatic digestion of the skin biopsies with Dispase II (Boehringer-Mannheim, Rotkreuz, Switzerland), fibroblasts were grown in DMEM containing 10% heat inactivated FCS. Fibroblasts from passages 3-9 were used for the experiments.

For exposure to hypoxia, fibroblasts were grown to 50-80% confluence and medium was changed 24 hours before initiation of the experiments. Fibroblasts were transferred into a hypoxic incubator (Forma Scientific, Illkirch, France) and exposed to a humidified atmosphere containing 5% CO\(_2\) and 1% O\(_2\) v/v (hypoxia, oxygen tension 7 mmHg) at 37\(^\circ\)C for 6 to 96 hours \(^3\). For control, cells were cultured under the same conditions except that the atmosphere contained 20% O\(_2\) v/v (normoxia, oxygen tension 140 mmHg). In experiments
under low growth factor conditions, medium was changed from DMEM/10% FCS to DMEM/1% FCS 24 hours before the experiments.

**Measurement of skin oxygenation**

Skin oxygenation was measured intradermally in 13 SSc patients and 5 healthy controls at the dorsal aspect of the forearm (midway between wrist and elbow) using a pO₂ histograph (Eppendorf, Germany). The healthy controls were age- and sex-matched with the SSc patients. To exclude circadian and seasonal influences on the results, all measurements were taken in the morning between 9 and 11 a.m. and during the same season (January-March). The pO₂ histograph consists of a microprocessor-guided polarographic electrode system and is considered the gold standard for the measurement of tissue oxygen tensions 4,5. Patients and controls were allowed to adapt to room temperature for at least 30 minutes. All measurements were performed with the subjects sitting in an upright position their forearm rested at a 90° angle. After local anesthesia with 1% Lidocaine (Sintetica, Switzerland), the skin was punctured with a needle, and the polarographic needle electrode was inserted 3-4 mm into the tissue. The electrode was then moved automatically through the tissue in steps of 0.4 mm with a forward movement of 0.7 mm followed immediately by a backward movement of 0.3 mm to minimize pressure artifacts. After every step, the pO₂ value was recorded. When 50 measurements were taken, the needle electrode was removed and the procedure was repeated in a different direction thereby recording 400 pO₂ values in a 4-cm² area of the middle forearm in each subject. To ensure that the pO₂ values were measured in the dermis, the location of the needle electrode was controlled by ultrasound. At the end of the experiment, blood gas analysis was performed by puncture of the A. radialis. In addition, blood pressure and hemoglobin were determined by standardized procedures. The mean duration of the experiment was 70 ± 10 minutes without differences between the groups. Initial experiments
with healthy volunteers and measurements at different days showed that the results were reproducible and were not influenced by the local anesthesia used.

**Western blot analysis and immunofluorescence**

Cultured cells were removed from the hypoxic incubator and rinsed quickly with ice-cold PBS. Cell-lysis buffer consisting of 10 mM Tris/HCl (pH 8.0), 1 mM EDTA (pH 8.0), 150 mM NaCl, 0.1% Nonidet P-40, 1 mM phenylmethylsulphonylfluoride (PMSF), 1 mM Na₃VO₄ and aprotinin/leupeptin/pepstatin A (all 1 µg/ml) was then immediately applied. After incubation on ice for 10 minutes, the homogenate was centrifuged at 1600 g and 4°C for 5 minutes. Nuclear extraction buffer containing 20 mM hydroxyethyl-piperazine-ethanesulfonic acid (HEPES, pH 7.5), 400 mM NaCl, 1 mM EDTA (pH 8.0), 1 mM dithiothreitol (DTT) and 1 mM PMSF was added to the pellet in a 1:1 ratio and incubated on ice for 15 minutes. After centrifugation (20 000g, 5 minutes, 4°C), supernatants were collected and stored at -80°C.

The Bradford assay (Biorad, Reinach, Switzerland) was used to determine protein concentrations. Thirty µg of nuclear extract per sample were separated by 7.5% SDS-PAGE and electrotransferred onto nitrocellulose membranes according to standard protocols. Protein transfer and equal loading were confirmed by Ponceau S staining. After blocking with 4% nonfat milk powder in phosphate-buffered saline (PBS) for 1 h, immunoblots were incubated with monoclonal mouse anti-HIF-1α mcg3 antibodies for 2 hours at a dilution of 1:500, and with horseradish peroxidase-conjugated goat anti-mouse antibodies (Promega/Catalys, Wallisellen, Switzerland) for 1 hour at a dilution of 1:10000. Chemiluminescence detection was performed by incubation with 100 nM Tris/HCl (pH 8.5), 2.65 mM H₂O₂, 0.45 mM luminol and 0.625 mM coumaric acid for 1 min, followed by exposure to X-ray films (SuperRX, Fuji, Dielsdorf, Switzerland). To verify equal loading of proteins, immunoblots were incubated with polyclonal rabbit anti-Sp1 antibodies (Santa Cruz,
Santa Cruz, CA; dilution 1:1000), followed by horseradish peroxidase-conjugated anti-rabbit antibodies (Sigma; dilution 1:10000).

For immunofluorescence, cells were cultured to sub-confluence in chamber slides, and exposed to hypoxia as described. Cells were then fixed immediately in freshly prepared 4% formaldehyde (pH 7.0) for 10 minutes. After incubation with 0.5% Triton X for 5 minutes and blocking with 10% FCS in PBS for 30 minutes, cells were incubated for 1 hour at 37°C with monoclonal mouse anti-HIF-1α mcg3 antibodies (dilution 1:10) or with isotype matched IgG antibodies (Dako, Zug, Switzerland) for control, followed by FITC conjugated goat anti-mouse antibodies (Jackson ImmunoResearch/Milan Analytica, LaRoche Switzerland, dilution 1:400) for 30 minutes at room temperature.

**Immunohistochemistry**

HIF-1α protein was detected by immunohistochemistry on paraffin embedded sections of skin biopsies from SSc patients and healthy controls as described recently. In brief, after deparaffinization and rehydration, endogenous peroxidase was blocked with 100% methanol/1%H2O2 at 4°C for 20 minutes. Antigen retrieval was performed using the Dako antigen retrieval system according to the instructions of the manufacturer. Sections were rinsed in PBS and incubated with 5% bovine serum albumin (BSA, Sigma) for 30 minutes and with 20% normal goat serum (NGS, Sigma) for 60 minutes at room temperature to block non-specific binding. Incubation with monoclonal mouse anti-HIF-1α antibodies (dilution 1:200, Novus, Littleton, CO, USA) was performed overnight at 4°C in 10% NGS/PBS. After rinsing in PBS/0.1% Triton and incubation with 20% NGS/PBS/0.1% Triton for 1 hour at room temperature, sections were exposed to biotinylated rat anti-mouse antibodies (dilution 1:300 in NGS/PBS/0.1% Triton, Dianova, Hamburg, Germany) for 1 hour. Sections were then incubated with alkaline phosphatase conjugated streptavidin (Vector, Burlingame, CA, USA) for 1 hour at room temperature, followed by visualization with Fast Red (Sigma). Control
experiments were performed with isotype-matched antibodies instead of the primary antibodies.

For the detection of Flk-1/VEGFR2, monoclonal mouse antibodies (Santa Cruz Biotechnology, Santa Cruz, USA) were used. Sections were exposed to microwave heating in 10 nM sodium citrate (pH 6.0) for antigen retrieval. Unspecific binding was blocked by incubation with 4% milk and 2% normal horse serum in Tris buffer (pH = 7.6) for 30 minutes at room temperature. Then, slides were incubated with anti-Flk-1/VEGFR-2 antibodies (1:50 dilution in Tris) for 2 hours, with secondary antibodies (goat anti-mouse IgG + IGM, H + L, diluted 1:400 in Tris; Jackson ImmunoResearch Laboratories, West Grove, USA) for 30 minutes and with APAAP complexes (1:50 dilution in Tris; Dako) for 30 minutes. All steps were performed at room temperature and with Tris/0.1% saponin as washing solution. Bound APAAP complexes were visualized with NBT/BCIP in 10% polyvinyl alcohol (molecular weight 70-100 kD). Negative controls consisted of isotype matched IgG<sub>1</sub> antibodies instead of the primary antibodies.

For Flt-1/VEGFR1 immunohistochemistry, a biotin-streptavidin based amplifying system (BioGenex, San Ramon, USA) with polyclonal rabbit IgG antibodies against flk-1 (Santa Cruz Biotechnology) was used. Paraffin embedded sections were processed as described above. After antigen retrieval, endogenous peroxidase activity was blocked with 3% H<sub>2</sub>O<sub>2</sub> for 5 minutes. Slides were incubated for 1 hour with anti-flt-1 antibodies (1:50 dilution in Tris) followed by incubation with biotinylated anti-immunglobulins for 20 minutes. Labeling was performed for 20 minutes using a horseradish peroxidase-conjugated streptavidin complex with high affinity for biotin (BioGenex). Antigens were visualized using aminoethylcarbazole (AEC) chromogen and hydrogen peroxide as substrate. For control, antibodies were preincubated with an excess of blocking peptide (10 times the concentration of the primary antibodies; Santa Cruz Biotechnology) for 1 hour at room temperature. This solution was then applied instead of the primary antibodies.
The results of each immunohistochemistry were quantified using a score ranging from no expression to high expression: (+) = weak staining of a few cells, + = <25% of cells stained positive, ++ = 25-50% positive cells, +++ > 50% positive cells.

For double-labeling experiments after in situ hybridization for VEGF, monoclonal mouse anti-CD68 antibodies (clone PG-M1, Dako, Glostrup, Denmark) were used. Unspecific binding was blocked by incubation with 4% milk and 2% normal horse serum in Tris buffer (pH = 7.6) for 30 minutes. Slides were incubated with anti-CD68 antibodies at a dilution of 1:60 in Tris buffer for 1 hour. Bound primary antibodies were linked with biotinylated anti-immunglobulins (BioGenex) for 20 minutes. Labeling was performed for 20 minutes using a horseradish peroxidase-conjugated streptavidin complex with high affinity for biotin (BioGenex). All steps were performed at room temperature. Antigens were visualized using aminoethylcarbazole (AEC) chromogen and hydrogen peroxide as substrate. In control experiments, matched mouse IgG isotypes were used instead of the primary antibodies.

Serial section of in situ hybridization for VEGF as well as of immunohistochemistry for Flk-1/VEGFR2 and Flt-1/VEGFR1 were stained with polyclonal rabbit anti-von Willebrand-Factor antibodies (A 0082, Dako). After blocking with 1% H2O2/0.01% NaN3 for 5 minutes, slides were incubated with the polyclonal rabbit anti-von Willebrand-Factor antibodies at a dilution of 1:2000 overnight at 4°C. The secondary antibody (biotin-labeled goat-anti-rabbit antibodies) was applied at a dilution of 1:1000 for 30 minutes at room temperature. Labeling was performed using the Vectastain ABC kit containing peroxidase (Vector Laboratories, Burlingame, CA, USA), and antigens were visualized with Nova RED (Vector Laboratories). In control experiments, irrelevant rabbit IgGs were used instead of the primary antibodies.

**Stimulation with PDGF and IL-1β**

To examine the effects of platelet-derived growth factor (PDGF) and interleukin-1β (IL-1β), which are known to be upregulated in the clinically involved skin of patients with SSc,
fibroblasts were cultured in 24-well plates. For stimulation with PDGF, fibroblasts were grown to confluence; DMEM/10%FCS was replaced with 1.5 ml DMEM without FCS and incubated for 24 hours. Recombinant PDGF-BB (R&D Systems, Abingdon, UK) stored at -80°C in stock concentration of 10 µg/ml in PBS/0.1% bovine serum albumin/4mM HCl was used for the experiments. Cells were washed with PBS, and recombinant PDGF-BB dissolved in 1.5 ml DMEM was applied at concentrations of 10 and 40 ng/ml on the culture dishes. Since initial studies had shown that the optimal induction of VEGF is seen after incubation for 24 hours, this duration was chosen for the experiments. Incubation with DMEM without PDGF, but with the same amounts of PBS/0.1% bovine serum albumin/4mM HCl, was used as negative control.

First experiments indicated that the induction of VEGF by interleukin 1β (IL-1β) was independent of whether the cells were cultured in DMEM/10%FCS or DMEM without FCS. Optimal results were obtained after 6 hours of incubation. Therefore, experiments were carried out in DMEM/10%FCS for 6 hours. Medium was changed 24 hours before the initiation of the experiments. Recombinant IL-1β (R&D Systems) was stored in aliquots at -80°C in stock concentration of 2 µg/ml in PBS/0.1% bovine serum albumin. After washing with PBS, 1.5 ml DMEM/10%FCS containing recombinant IL-1β at concentrations of 1 pg/ml, 10 pg/ml and 100 pg/ml were applied on the culture dishes. Controls consisted of DMEM/10%FCS without IL-1β, but with the same amounts of PBS/0.1% bovine serum albumin used for the stimulation. Co-stimulation experiments were performed with 40 ng/ml PDGF and 100 pg/ml IL-1β in DMEM without FCS for 24 hours under the same conditions as described above.
### Tables

<table>
<thead>
<tr>
<th></th>
<th>Numbers</th>
<th>Gender (F/M)</th>
<th>Age (median/range)</th>
<th>Disease subset (diffuse/limited)</th>
<th>Disease duration (median/range)</th>
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<td>3 years (1 – 43)</td>
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<td><strong>PO$_2$ measurements</strong></td>
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<td>62 years (51-70)</td>
<td>3/10</td>
<td>6 years (2-26)</td>
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**Online Table 1:** Characteristics of SSc patients. F = female, M = male. The disease subset was determined according to the criteria proposed by LeRoy et al $^9$. Disease duration was measured from the onset of the first non-Raynaud symptoms attributable to SSc.
Online Table 2: Semiquantitative analyses of the HIF-1α expression in skin specimens from normal controls and patients with SSc. Numbers of patients/controls for each histological score are shown. In all skin specimens from healthy controls, an expression of HIF-1α could be detected in the epidermis. In the majority of SSc patients, the expression of HIF-1α was weak or absent in the epidermis and no expression could be detected in the dermis, contrasting with the abundant expression of VEGF in the same patients (see Figures 1 and 4).

(+): weak staining of a few keratinocytes, +: <25% of keratinocytes stained positive, ++: 25-50% positive, +++: >50% positive

<table>
<thead>
<tr>
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<th>Dermis</th>
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<tr>
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<td></td>
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<tr>
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Online Table 3: Semi-quantitative analyses of the expression of flt-1/VEGFR1 and flk-1/VEGFR2 in involved (fibrotic) skin biopsies from SSc patients and healthy controls. Numbers of patients/controls for each histological score are shown. Similar to VEGF itself, the expression of flt-1/VEGFR1 as well as flk-1/VEGFR2 showed a strong upregulation in SSc patients compared to healthy controls (see also Figure 6 of the print-version and Figures 3 and 4 of the online supplement). (+) = weak staining of a few endothelial cells, + = <25% of endothelial cells stained positive, ++ = 25-50% positive, +++ > 50% positive
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**Online Table 4:** Semi-quantitative analyses of the expression of flt-1/VEGFR1 and flk-1/VEGFR2 in involved skin and non-involved skin biopsies. Immunohistochemistry was performed on fresh-frozen samples. Numbers of patients/controls for each histological score are shown. Expression of both VEGF-Receptors was lower in non-involved (non-fibrotic) skin biopsies than in involved (fibrotic) skin biopsies of the same patients. (+) = weak staining of a few endothelial cells, + = <25% of endothelial cells stained positive, ++ = 25-50% positive, +++ > 50% positive
Figure legends

Figure 1
Double labeling with anti-CD68 antibodies by immunohistochemistry (brown signal, AEC development) after in situ hybridization for VEGF (dark blue signal, NBT/BCIP development) on a skin section of a SSc patient. Coexpression with CD68 indicates that monocytes/macrophages express VEGF mRNA in the SSc skin. Skin biopsy of clinically non-affected skin, fresh-frozen biopsy, magnification x 630.

Figure 2
Serial sections of a skin biopsy from a patient with SSc. A, In situ hybridization showing expression of VEGF mRNA in smaller vessels (dark blue-signal, NBT/BCIP development). B, Coexpression with the endothelial marker von Willebrand-Factor (brown signal, Nova Red development) in the serial section of the same biopsy indicating that VEGF mRNA is expressed by endothelial cells. Skin biopsy of affected skin, paraffin-embedded biopsy, magnification x 630.

Figure 3
Serial sections of a skin biopsy from another patient with SSc. A, Immunohistochemistry with anti-flk-1/VEGFR2 antibodies showing expression of flk-1/VEGFR2 in numerous vessels (arrows, dark blue-signal, NBT/BCIP development). B, Serial section of the same biopsy. Immunohistochemistry with antibodies against the endothelial marker von Willebrand-Factor (arrows, brown signal, Nova Red development). Coexpression of flk-1/VEGFR2 and von Willebrand-Factor indicates that VEGF mRNA is expressed by endothelial cells. Skin biopsy of affected skin, paraffin-embedded biopsy, magnification x 100.
Figure 4

Serial sections of a skin biopsy from an additional patient with SSc. A, Immunohistochemistry with anti-flt-1/VEGFR1 antibodies showing expression of flt-1/VEGFR1 in vessels (arrows, brown signal, AEC development). B, Serial section of the same biopsy. Immunohistochemistry with antibodies against the endothelial marker von Willebrand-Factor (arrows, dark brown signal, Nova Red development). Coexpression of flt-1/VEGFR1 and von Willebrand-Factor indicates that VEGF mRNA is expressed by endothelial cells. Skin biopsy of affected skin, fresh-frozen biopsy, magnification x 200.
References


Fig. 1 online supplement
Fig. 2 online supplement
Fig. 3 online supplement
Fig. 4 online supplement