Overexpression of VEGF$_{165b}$, an Inhibitory Splice Variant of Vascular Endothelial Growth Factor, Leads to Insufficient Angiogenesis in Patients With Systemic Sclerosis

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Rationale: Systemic sclerosis (SSc) is characterized by widespread microangiopathy, fibrosis, and autoimmunity. Despite the lack of angiogenesis, the expression of vascular endothelial growth factor A (VEGF) was shown to be upregulated in SSc skin and circulation; however, previous studies did not distinguish between proangiogenic VEGF$_{165}$ and antiangiogenic VEGF$_{165b}$ isoforms, which are generated by alternative splicing in the terminal exon of VEGF pre-RNA.

Objective: We investigated whether VEGF isoform expression could be altered in skin and circulation of patients with SSc.

Methods and Results: Here, we show that the endogenous antiangiogenic VEGF$_{165b}$ splice variant is selectively overexpressed at both the mRNA and protein levels in SSc skin. Elevated VEGF$_{165b}$ expression correlated with increased expression of profibrotic transforming growth factor-$\beta$1 and serine/arginine protein 55 splicing factor in keratinocytes, fibroblasts, endothelial cells, and perivascular inflammatory cells. Circulating levels of VEGF$_{165b}$ were significantly higher in patients with SSc than in control subjects. Microvascular endothelial cells (MVECs) isolated from SSc skin expressed and released higher levels of VEGF$_{165b}$ than healthy MVECs. Transforming growth factor-$\beta$1 upregulated the expression of VEGF$_{165b}$ and serine/arginine protein 55 in both SSc and healthy MVECs. In SSc MVECs, VEGF receptor-2 was upexpressed, but its phosphorylation was impaired. Recombinant VEGF$_{165b}$ and SSc-MVEC–conditioned medium inhibited VEGF$_{165}$-mediated VEGF receptor-2 phosphorylation and capillary morphogenesis in healthy MVECs. The addition of anti-VEGF$_{165b}$ blocking antibodies abrogated the antiangiogenic effect of SSc-MVEC–conditioned medium. Capillary morphogenesis was severely impaired in SSc MVECs and could be ameliorated by treatment with recombinant VEGF$_{165}$ and anti-VEGF$_{165b}$ blocking antibodies.

Conclusions: In SSc, a switch from proangiogenic to antiangiogenic VEGF isoforms may have a crucial role in the insufficient angiogenic response to chronic ischemia. (Circ Res. 2011;109:00-00.)

Key Words: systemic sclerosis • scleroderma • peripheral vascular diseases • angiogenesis • antiangiogenic agents • vascular endothelial growth factor A

Systemic sclerosis (SSc, or scleroderma) is a chronic, multisystem connective tissue disorder characterized by widespread microangiopathy, fibrosis, and autoimmunity that affects the skin and various internal organs, including the heart, lung, kidney, and gastrointestinal tract. Increasing clinical and pathogenetic evidence indicates that vascular damage is a primary event in SSc and that it is characterized by an increase in circulating markers of endothelial cell activation and injury and by vascular tone dysfunction. Nailfold capillaroscopy shows a variety of morphological changes, including enlarged, giant, and bushy capillaries, microhemorrhages, and a variable loss of capillaries with formation of avascular areas. Chronic tissue ischemia and lack of angiogenesis may lead to severe peripheral vascular complications such as digital ulceration and gangrene. It is well known that tissue ischemia and hypoxia usually lead to the expression of proangiogenic growth factors, which then initiate angiogenic sprouting from preexisting microvessels.
sels by inducing vasodilation, proliferation, and migration of endothelial cells, invasion of the surrounding stroma by endothelial cells, and stabilization of the vascular lumen. Vascular endothelial growth factor A (VEGF-A, hereafter referred to as VEGF) is one of the major proangiogenic regulators and is found to be overexpressed in most physiological and pathological angiogenic states. Several studies have shown that VEGF expression is markedly increased in different cell types both in the epidermis and dermis of patients with SSc. VEGF exerts its biological functions by binding to the tyrosine kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), both of which are upregulated on dermal microvascular endothelial cells in SSC-affected skin. Moreover, a number of studies demonstrated that circulating levels of VEGF are significantly increased in SSc patients throughout different disease stages and correlate with organ manifestations. However, in SSc, there is no evidence of effective angiogenesis, and the disease evolves toward a progressive loss of capillary vessels. Collectively, this scenario has been regarded as a paradox. Indeed, the cellular and molecular mechanisms responsible for the lack of a sufficient angiogenic response to chronic ischemia in the presence of elevated proangiogenic mediators remain largely unknown.

The VEGF gene consists of 8 exons separated by 7 introns and spans approximately 14 kb. VEGF pre-mRNA is differentially spliced to form mRNAs that encode at least 6 isoforms that have been widely studied and accepted as proangiogenic and vascular permeability factors. The main isoform is VEGF 165, which has 165 amino acids in the final structure. However, recent evidence indicates that 2 families of VEGF proteins are formed by alternative splice-acceptor-site selection in the 3′ untranslated region within the terminal exon 8 to give different C-terminal sequences that differ in only 6 amino acids. Proximal splice-acceptor-site selection in exon 8 results in classic proangiogenic VEGF isoforms that contain the exon 8α sequence, whereas distal splice-acceptor-site selection results in antiangiogenic VEGF isoforms that contain the exon 8b sequence (where xxx denotes the amino acid number). The proangiogenic isoforms encode a terminal 6-amino acid sequence of Cys-Asp-Lys-Pro-Arg-Arg, whereas the antiangiogenic isoforms encode Ser-Leu-Thr-Arg-Lys-Asp. The most widely studied VEGF 165b isoform is VEGF 165b, but other isoforms have also been identified at both the mRNA and protein levels. VEGF 165b transcript and protein are widely expressed in most healthy human tissues and in nonangiogenic tissues, although unlike other VEGF isoforms, VEGF 165b is downregulated in angiogenic conditions, such as renal, prostate, colon, and skin cancers and proliferative diabetic retinopathy. In contrast, in nonangiogenic pathological conditions, such as glaucoma, the antiangiogenic VEGF isoforms are upregulated. VEGF 165b protein was shown to significantly and dose dependently inhibit VEGF 165-mediated proliferation and migration of endothelial cells, vasodilation of mesenteric arteries, and angiogenesis in 2 different models of VEGF 165b-driven blood vessel growth. Furthermore, it has been shown to inhibit hypoxia-driven angiogenesis in the retina and the growth of a variety of human tumor xenografts in mice. Indeed, the dimerization and receptor-binding domains are still present in VEGF 165b, and hence, it acts as a competitive inhibitor of VEGF 165; it binds to the receptor but does not stimulate the full tyrosine phosphorylation of the VEGFR-2 and downstream signaling activated by VEGF 165. VEGF 165b therefore appears to be an endogenous antiangiogenic agent formed by alternative splicing.

Recent studies have shown that regulation of alternative splicing by growth and splicing factors is a key event in determining the relative expression of proangiogenic versus antiangiogenic VEGF isoforms. VEGF 165b expression is upregulated by some growth factors, such as the profibrotic transforming growth factor-β (TGF-β) cytokine, through the activation of p38 mitogen-activated protein kinase (MAPK) and the CDC-like kinase Clk-1/Sty splicing factor kinase family pathways. In this context, the mRNA-binding protein serine/arginine protein 55 (SRp55, also known as splicing factor arginine-serine-rich 6 [SFRS6]) has been identified as a key regulatory splicing factor that binds to VEGF pre-RNA in the exon 8 region that promotes distal splice-site selection and subsequent upregulation of exon 8b–containing VEGF 165b.

Because the amino acid structure of antiangiogenic VEGF 165b is 95% to 96% identical to that of VEGF 165, all previous studies that have investigated VEGF expression in SSc could not distinguish between the proangiogenic and antiangiogenic VEGF isoforms. Indeed, those studies were conducted before the discovery of antiangiogenic VEGF isoforms or used in situ hybridization riboprobes, antibodies, and ELISAs that pick up both VEGF 165 and VEGF 165b isoforms. We therefore undertook the present study to investigate whether the expression of VEGF splicing variants could be altered in the skin and circulation of patients with SSc.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org and includes detailed information regarding the following: characteristics of patients with SSc and healthy subjects used for collection of forearm skin biopsy samples, plasma samples, and isolation of dermal microvascular endothelial cells (MVECs); cell culture and stimulation protocols; semiquantitative reverse transcription–polymerase chain reaction (RT-PCR); quantitative real-time RT-PCR; antibodies; immunoprecipitation and Western blotting on tissue and cell protein lysates; immunohistochemistry and confocal laser scanning microscopy; ELISAs on plasma samples and culture supernatants; in vitro capillary morphogenesis assay; and statistical analysis.
Results

The Antiangiogenic VEGF_{165b} Splice Variant Is Selectively Upregulated at Both mRNA and Protein Levels in Skin Biopsy Samples From Patients With SSc

Expression of the VEGF_{165b} splice variant was investigated in full-thickness skin biopsy samples obtained from the clinically involved skin of one-third of the distal forearm of 35 patients with SSc and from the same forearm region of 23 age- and sex-matched healthy donors. All patients fulfilled the criteria for SSc as suggested by LeRoy and Medsger.\(^2^6\) Fifteen patients had limited cutaneous SSc (lcSSc) and 20 had diffuse cutaneous SSc (dcSSc) according to the criteria proposed by LeRoy et al.\(^2^7\) Patients were further classified as being in the early (n=21) or late (n=14) phase of SSc according to disease duration and to the clinical and pathological stage of skin involvement, as detailed in the Online Data Supplement. The characteristics of patients used for collection of skin biopsy samples are listed in Online Table I.

After mRNA extraction from skin biopsy samples and cDNA synthesis, cDNA was amplified with exon 4/exon 8b–specific primers that detect the VEGF_{165b} splice variant only or exon 4/exon 8a–specific primers that pick up proangiogenic VEGF_{165} but not antiangiogenic VEGF_{165b}. Because previous studies have shown that VEGF_{165b} mRNA is downregulated whereas VEGF_{165} mRNA is upregulated in renal cancer,\(^7\) we used renal cell carcinoma samples as an additional disease control group for the RT-PCR assay. As shown in Figure 1A, VEGF_{165b} mRNA was detected in all skin biopsy samples from SSc patients and healthy subjects, whereas VEGF_{165} mRNA was very weakly expressed or almost undetectable in both groups. As expected in renal cell carcinoma, VEGF_{165b} mRNA was weakly expressed, whereas VEGF_{165} mRNA was highly expressed. VEGF_{165b} mRNA was found to be upregulated in the majority of SSc skin samples compared with healthy control subjects (Figure 1A). Quantitative real-time RT-PCR confirmed that VEGF_{165b} transcript levels were significantly increased in SSc skin compared with control skin (P<0.001; Figure 1B).

To analyze VEGF_{165b} protein expression, skin sections from patients with SSc and healthy subjects were immunostained with a mouse monoclonal antibody that specifically detects the VEGF_{165b} splice variant but not proangiogenic VEGF_{165}.\(^1^1,\(^1^2\) This antibody is raised to the 9 C-terminal amino acids of human VEGF_{165b}, the final 6 of which are specific to VEGF_{165b}.\(^1^1\) To confirm antibody specificity, the anti-VEGF_{165b} antibody was used to probe a blot of an SDS–polyacrylamide gel loaded with recombinant human VEGF_{165b} and VEGF_{165}. Figure 2A shows that this antibody specifically recognized VEGF_{165b} and did not detect VEGF_{165}. In contrast, anti-pan-VEGF antibodies detect both VEGF_{165b} and VEGF_{165} isoforms.

Consistent with recent findings,\(^1^4\) constitutive expression of VEGF_{165b} was observed in both the epidermis and dermis of healthy control subjects (Figures 2B and 2C). Whereas in the majority of healthy subjects, the expression of VEGF_{165b} ranged from weak to moderate (Figure 2B), an intense immunopositivity was found in keratinocytes, dermal fibroblasts, and capillary vessels of 6 of 23 control samples (Figure 2C). A marked upregulation of VEGF_{165b} was found in the majority of affected skin biopsy samples from patients with SSc (Figures 2D–H). In both early and late SSc skin, an enhanced expression of VEGF_{165b} could be detected in epidermal keratinocytes and in the papillary and reticular dermis, where it was found in fibroblasts and microvascular endothelial cells (Figures 2D–F). In early SSc skin, perivascular inflammatory cells infiltrating the dermis were also strongly immunopositive for VEGF_{165b} (Figure 2D). Furthermore, in early SSc, endothelial cells and vascular smooth muscle cells of dermal arterioles and large inflammatory...
infiltrates found in the hypodermis also showed a strong expression of VEGF<sub>165b</sub> (Figures 2G and 2H). Double-labeling experiments with an antibody against the pan-endothelial cell marker CD31 confirmed the expression of VEGF<sub>165b</sub> in endothelial cells and perivascular inflammatory cells (Figures 2I and 2J). Semiquantitative analysis of immunofluorescent staining intensity on skin sections showed that VEGF<sub>165b</sub> expression was increased significantly in both the epidermis and dermal cells of patients with SSc (both \( P < 0.001 \) compared with healthy control subjects; Figure 2K).

The pattern of VEGF<sub>165b</sub> expression observed in the skin of SSc patients was very similar to that described previously for VEGF in different studies. However, previous studies detected VEGF in SSc skin specimens using in situ hybridization riboprobes or antibodies that recognize both VEGF<sub>165</sub> and VEGF<sub>165b</sub> isoforms. Therefore, we immunostained serial skin sections from SSc patients with the VEGF<sub>165b</sub>-specific antibody and 3 different pan-VEGF antibodies that react with both VEGF<sub>165</sub> and VEGF<sub>165b</sub> (see the Online Data Supplement). Staining with the VEGF<sub>165b</sub>-specific antibody revealed that in both normal and SSc skin, VEGF<sub>165b</sub> was expressed in the same cells as the pan-VEGF stain, and with a very similar intensity (Figure 3A). In fact, in a semiquantitative analysis, no significant difference in immunofluorescent staining intensity could be found. Each of the 3 pan-VEGF antibodies gave similar results. Moreover, double immunostaining with mouse anti-VEGF<sub>165b</sub> antibody and rabbit anti-pan-VEGF antibody revealed that the 2 markers were almost completely colocalized in the same tissue structures (Figure 3B).

Western blotting was used to evaluate VEGF<sub>165b</sub> and pan-VEGF expression levels in crude protein extracts from whole skin samples. Both VEGF<sub>165b</sub>-specific antibody and pan-VEGF antibodies detected a prominent protein band with...
**Figure 3.** VEGF<sub>165b</sub> and pan-VEGF expression in skin biopsy samples. A, Serial skin sections from SSc patients immunostained with anti-VEGF<sub>165b</sub>-specific antibodies (upper panels) and anti-pan-VEGF antibodies (lower panels). In epidermis (e) and dermis, VEGF<sub>165b</sub>-specific and pan-VEGF stains showed similar intensity in the same tissue structures. Asterisks indicate a dermal perivascular inflammatory infiltrate. B, Double immunostaining for VEGF<sub>165b</sub> (green) and pan-VEGF (red) in SSc skin. Original magnification, ×63. C, Total protein extracts of SSc and control skin were probed with anti-VEGF<sub>165b</sub>-specific and anti-pan-VEGF antibodies. Results of a representative experiment are shown. SSc 1 to 3 indicates early SSc skin; SSc 4 and 5, late SSc skin. D, Densitometric analysis of blot bands. Data are mean±SD of optical density in arbitrary units (a.u.). *P<0.001 vs VEGF<sub>165b</sub> in control. **P<0.001 vs pan-VEGF in control. E, Different concentrations of recombinant human (rh) VEGF<sub>165b</sub> were probed with anti-VEGF<sub>165b</sub>-specific and anti-pan-VEGF antibodies.
a molecular weight of 23 kDa, which is consistent with the monomeric forms of both VEGF_{165}b and VEGF_{165} (Figure 3C). Moreover, both antibodies revealed a smaller band with a molecular weight of 46 kDa, consistent with the persistence of small amounts of VEGF_{165}b and VEGF_{165} homodimers under standard reducing conditions (Figure 3C). In accordance with our findings, it has been reported previously that the anti-VEGF_{165}b antibody recognizes both the VEGF_{165}b monomer and dimer in Western blotting experiments. In additional Western blotting experiments with stronger reducing conditions, only the VEGF_{165}b monomer (23 kDa) could be detected (Online Figure I). Western blotting results paralleled those obtained by immunohistochemistry. In skin from patients with SSC, there was a statistically significant increase in both VEGF_{165}b and pan-VEGF protein expression levels compared with skin from healthy subjects (both P<0.001; Figure 3D). Indeed, blot bands obtained with the VEGF_{165}b-specific antibody were equivalent in intensity to those obtained with pan-VEGF antibodies in the same individual skin samples (Figure 3C). In fact, densitometric analysis did not reveal any significant difference between VEGF_{165}b and pan-VEGF protein levels in either SSC or control skin samples (Figure 3D). In addition, in a blot of an SDS–polyacrylamide gel loaded with different concentrations of recombinant human VEGF_{165}b, anti-VEGF_{165}b and anti-pan-VEGF antibodies detected bands of comparable intensity for the same amounts of loaded protein (Figure 3E). This indicates that the antibodies recognize VEGF_{165}b protein with the same affinity. Moreover, the preincubation of anti-VEGF_{165}b antibody with an excess of recombinant human VEGF_{165}, abolished the blot stainings, which further indicates that the anti-VEGF_{165}b antibody specifically detected VEGF_{165}b in skin protein extracts assayed in Western blotting experiments (Online Figure I). Collectively, these data indicate that almost all the VEGF protein detectable in both SSC and control skin specimens was the antiangiogenic VEGF_{165}b isoform.

No significant difference in skin expression of VEGF_{165}b was found between patients with lcSSc and those with dcSSc. Instead, VEGF_{165}b protein levels were higher in early SSC than in late SSC skin (Figure 3C). This was consistent with the immunohistochemical findings. In fact, in addition to keratinocytes, endothelial cells, and fibroblasts, infiltrating inflammatory cells also showed strong expression of VEGF_{165}b in early SSC skin (Figures 2D, 2H, and 2J).

**VEGF_{165}b Levels Are Raised in the Circulation of Patients With SSC**

Blood samples were obtained from 61 patients with SSC (36 lcSSc and 25 dcSSc; 28 early and 33 late SSC phase) and 30 age- and sex-matched healthy subjects. The characteristics of patients used for collection of blood samples are listed in Online Table II. Circulating levels of VEGF_{165}b were measured in SSC and control plasma with a quantitative sandwich ELISA that does not cross-react to proangiogenic VEGF isoforms (see the Online Data Supplement).

Plasma levels of VEGF_{165}b were significantly higher in SSC patients (median 288.76 pg/mL, range 56.43 to 1056.0 pg/mL) than in healthy control subjects (median 137.34 pg/mL, range 0 to 351.75 pg/mL; P<0.001; Figure 4A). No significant difference was detected between lcSSc (median 242.42 pg/mL, range 75.21 to 1056.0 pg/mL) and dcSSc (median 299.69 pg/mL, range 56.43 to 993.72 pg/mL). Circulating levels of VEGF_{165}b were significantly higher in both lcSSc and dcSSc than in control subjects (both P<0.002; Figure 4A). Plasma VEGF_{165}b was increased significantly in both the early (median 305.14 pg/mL, range 69.58 to 1056.0 pg/mL) and late (median 249.63 pg/mL, range 56.43 to 985.79 pg/mL) SSC phase compared with healthy control subjects (P<0.001 and P=0.004, respectively), whereas it was not different between early and late SSC (Figure 4B).

We also analyzed circulating levels of pan-VEGF in SSC and control subjects using an ELISA that recognizes both VEGF_{165} and VEGF_{165}b. Pan-VEGF levels were in the range of those detected for VEGF_{165}b with VEGF_{165}b-specific ELISA. Pan-VEGF levels were significantly raised in SSC patients (median 323.13 pg/mL, range 67.94 to 1291.08 pg/mL) compared with healthy subjects (median 153.87 pg/mL, range 0 to 433.50 pg/mL; P<0.001; Figure 4C). Pan-VEGF was significantly higher in the circulation of both lcSSc (median 278.85 pg/mL, range 87.24 to 1193.71 pg/mL) and dcSSc (median 387.81 pg/mL, range 67.94 to 1291.08 pg/mL) than in control subjects (P=0.002 and P<0.001, respectively; Figure 4C), whereas it was not different between lcSSc and dcSSc. No significant difference was detected between the early (median 368.37 pg/mL, range 92.15 to 1178.24 pg/mL) and late (median 314.22 pg/mL, range 67.94 to 1291.08 pg/mL) SSC phase. Circulating pan-VEGF levels were significantly elevated in both early and late SSC compared with control subjects (P<0.001 and P<0.001, respectively; Figure 4D). Collectively, the findings of pan-VEGF ELISA paralleled those obtained with VEGF_{165}b-specific ELISA and indicated that the majority of circulating VEGF in both SSC and control subjects was the VEGF_{165}b splice variant.

**In SSC Skin, VEGFR-2 Is Upregulated and Coexpressed With VEGF_{165}b in Endothelial Cells**

Recent studies have shown that VEGF_{165}b binds to VEGFR-2 with the same affinity as VEGF_{165} but does not activate proangiogenic signals. We therefore performed double immunohistochemistry with antibodies against VEGF_{165}b and VEGFR-2 on skin sections from SSC patients and control subjects. In control skin, very weak or no expression of VEGFR-2 could be observed (Figure 5A). Consistent with previous reports, the expression of VEGFR-2 was found to be significantly upregulated in SSC skin biopsy samples (P<0.001 compared with control subjects), and no dermal structures other than endothelial cells expressed VEGFR-2 (Figures 5B–D). In both the papillary and reticular dermis of SSC skin, VEGF_{165}b and VEGFR-2 were upregulated and coexpressed in endothelial cells (Figures 5B and 5C). Quantitative real-time RT-PCR confirmed that VEGFR-2 transcript levels were significantly increased in SSC compared with control skin (P<0.001; Figure 5E).
Upregulated VEGF<sub>165b</sub> Correlates With Increased Expression of TGF-β1 and SRp55 Splicing Factor in SSc Skin

It has been demonstrated recently that growth factors, especially TGF-β1, and splicing factors, particularly SRp55, can preferentially select for the distal splice site in the VEGF pre-mRNA terminal exon 8, thus resulting in upregulation of the VEGF<sub>165b</sub> isoform. We therefore performed double immunohistochemistry to investigate whether the expression of VEGF<sub>165b</sub> in affected skin from patients with SSc could be correlated with the expression of TGF-β1 and SRp55.

Consistent with previous findings, we observed a strong expression of profibrotic TGF-β1 in the affected skin of SSc patients (Figure 6). The expression of TGF-β1 correlated well with that of VEGF<sub>165b</sub>. Indeed, the 2 cytokines were coexpressed in different cell types, including keratinocytes, dermal fibroblasts, endothelial cells, and perivascular inflammatory cells (Figure 6).

Although in the majority of control skin specimens, the expression of SRp55 ranged from weak to moderate (Figure 7A; Online Figure II, A), an intense immunopositivity was found in the 6 of 23 control subjects who also showed increased VEGF<sub>165b</sub> expression. A strong upregulation of SRp55 was observed in the affected skin biopsy samples from SSc patients (Figure 7A; Online Figure II, A). In SSc skin, upregulated SRp55 and VEGF<sub>165b</sub> were coexpressed in both the epidermis and dermis, with an intense double-immunopositive signal in keratinocytes, fibroblasts, endothelial cells, and perivascular infiltrating inflammatory cells (Figure 7A; Online Figure II, A). Western blotting analysis on skin tissue homogenates revealed that SRp55 expression levels were increased significantly in SSc patients (P<0.001 compared with control subjects; Figure 7B and Online Figure II, B). Moreover, SRp55 protein levels were higher in early than in late SSc skin. No significant difference in SRp55 expression was found between skin specimens from patients with lcSSc and those with dcSSc.

Expression of VEGF<sub>165b</sub> and SRp55 Is Increased and Modulated by TGF-β1 in Dermal MVECs Isolated From SSc Skin

Dermal MVECs were isolated from forearm biopsy samples of involved skin from 6 patients with dcSSc and from 6 healthy subjects (see the Online Data Supplement). Pan-endothelial cell marker CD31 staining yielded positive results in more than 90% of the cell population. No difference in cell density, once at confluence, was observed between healthy MVECs and SSc MVECs, which allowed us to perform experiments in conditions of similar cell density.

The basal protein expression of VEGF<sub>165b</sub> and SRp55 was higher in SSc MVECs than in healthy MVECs (Figure 8A). As determined by ELISA, SSc MVECs constitutively released significantly higher levels of VEGF<sub>165b</sub> in culture supernatant compared with healthy MVECs (P<0.001). After TGF-β1 stimulation, the levels of VEGF<sub>165b</sub> in culture supernatants were significantly increased in both SSc
and healthy MVECs (both \( P < 0.001 \) compared with unstimulated cells; Figure 8A).

**Increased Levels of Antiangiogenic VEGF\(_{165b}\) in SSc MVECs Lead to Impaired VEGF-2 Phosphorylation and Downstream Signaling and Result in Defective Angiogenesis**

Consistent with findings of immunohistochemical staining on skin sections, the basal protein expression of VEGF-2 was found to be increased in SSc MVECs compared with healthy MVECs (Figure 8B); however, immunoprecipitation with antiphosphotyrosine antibodies and subsequent Western blotting analysis with anti-VEGFR-2 antibodies revealed that only a small amount of VEGF-2 was phosphorylated in SSc MVECs. Indeed, levels of phosphorylated VEGF-2 were strongly reduced in SSc MVECs compared with healthy MVECs (Figure 8B). In agreement with previous reports,\(^{11}\) we found that healthy MVECs had a basal level of VEGF-2 phosphorylation that was strongly increased by recombinant VEGF\(_{165}\) and decreased by VEGF\(_{165b}\). Moreover, VEGF\(_{165b}\) was able to inhibit VEGF\(_{165}\)-mediated VEGF-2 phosphorylation (Figure 8B). Treatment of healthy MVECs with SSc-MVEC–conditioned medium decreased VEGF-2 phosphorylation with basal cells. However, the addition of anti-VEGF\(_{165b}\) blocking antibodies to SSc-MVEC–conditioned medium not only abrogated this inhibitory effect but also resulted in increased VEGF-2 phosphorylation levels in healthy MVECs (Figure 8B).

We then investigated the downstream signaling pathways that are activated in MVECs after VEGF-2 phosphorylation. SSc MVECs had lower levels of phosphorylated extracellular signal-regulated kinase 1/2 (phospho-ERK1/2) MAPK than healthy MVECs (Online Figure III). Instead, phosphorylation of Akt was not different between SSc MVECs and healthy MVECs. Although treatment with proangiogenic VEGF\(_{165}\) resulted in a strong increase of phospho-ERK1/2 in healthy MVECs, this was not the case for incubation with VEGF\(_{165b}\) alone or in combination with VEGF\(_{165}\) (Online Figure III). Incubation with SSc-MVEC–conditioned medium reduced the levels of phospho-ERK1/2, whereas the addition of anti-VEGF\(_{165b}\) blocking antibodies to SSc-MVEC–conditioned medium resulted in increased phospho-ERK1/2 in healthy MVECs (for the comparison with untreated cells, see Online Figure III). Instead, treatment with VEGF\(_{165b}\) or SSc-MVEC–conditioned medium did not affect phosphorylation of Akt in healthy MVECs.

Using an in vitro capillary morphogenesis assay, we observed that the ability of healthy MVECs to form tubular structures on Matrigel was strongly potentiated by treatment with recombinant VEGF\(_{165}\), whereas it was inhibited...
by VEGF<sub>165b</sub> (Figure 8C). In addition, VEGF<sub>165b</sub> was able to inhibit VEGF<sub>165</sub>-mediated sprouting angiogenesis (Figure 8C). SSc-MVEC-conditioned medium also showed an inhibitory effect on healthy MVEC angiogenesis. Instead, the addition of anti-VEGF<sub>165b</sub> blocking antibodies to SSc-MVEC-conditioned medium resulted in stimulation of healthy MVEC angiogenesis (Figure 8C). As shown previously, capillary morphogenesis was severely impaired in SSc MVECs. Compared with healthy MVECs, after 6 hours of plating on Matrigel, SSc MVECs were unable to spontaneously produce elongated processes and to organize as branching cords. After 24 hours, SSc MVECs formed aggregates of cells but were unable to spontaneously organize into a honeycomb morphological pattern, producing only an incomplete endothelial network (Figure 8C). Stimulation of SSc MVECs with recombinant VEGF<sub>165</sub> at 10 ng/mL resulted in a slight promotion of angiogenesis. Instead, the use of recombinant VEGF<sub>165</sub> at 50 ng/mL significantly increased capillary morphogenesis in SSc MVECs. The combination of proangiogenic VEGF<sub>165</sub> (50 ng/mL) and anti-VEGF<sub>165b</sub> blocking antibodies further promoted the sprouting angiogenic response in SSc MVECs, reaching levels comparable to those observed in basal healthy MVECs (Figure 8C).

**Discussion**

Our data show for the first time that the endogenous antiangiogenic VEGF<sub>165b</sub> splice variant is selectively upregulated in SSc skin and circulation and that this property is retained in cultured dermal MVECs isolated from patients’ skin. Moreover, we provide evidence that profibrotic TGF-β1 and SRp55 splicing factor may contribute to the switch from proangiogenic to antiangiogenic VEGF isoforms and that constitutive overexpression of antiangiogenic VEGF<sub>165b</sub> leads to impaired VEGFR-2 downstream signaling and capillary morphogenesis in SSc MVECs. Finally, our in vitro findings suggest that neutralization of elevated VEGF<sub>165b</sub> might represent a potential strategy to promote an effective angiogenic response in patients with SSc.

Since its discovery in 1983, VEGF has raised interest because of its role in neovascularization in a variety of physiological and pathological processes, such as the female reproductive cycle, wound healing, cancers, myocardial ischemia, and rheumatoid arthritis and other autoimmune diseases. VEGF participates in many different steps of angiogenesis, including initial vasodilation, endothelial cell permeability, remodeling of the perivascular matrix, and induction of proliferation and migration of endothelial cells. These proangiogenic effects are mediated principally by activation of VEGFR-2. In fact, phosphorylation of ERK1/2 MAPK, Akt, and p38 MAPK by VEGFR-2 activation appears to be necessary for the promotion of endothelial cell proliferation, resistance to apoptosis, migration through extracellular matrix, and formation of a vascular lumen. Endothelial cells also express VEGFR-1, but its specific role in endothelial physiology is less well defined and somewhat controversial. VEGF is highly upregulated in all cases of pathological angiogenesis described to date. VEGF<sub>165</sub>, VEGF<sub>189</sub>, and VEGF<sub>121</sub> are commonly overexpressed, but VEGF<sub>165b</sub> appears to predominate quantitatively and functionally in most angiogenic states.

Recent studies, however, demonstrated that the VEGF primary transcript can be alternatively spliced in terminal exon 8, producing mRNA splice variants that are translated to proangiogenic and antiangiogenic isoforms. These splice variants differ by 6 amino acids at the C-terminus that switch the properties of these variants from proangiogenic to antiangiogenic activity. The predominant antiangiogenic isoform is VEGF<sub>165b</sub>, which binds to VEGFR-2 with the same affinity as VEGF<sub>165</sub> but does not activate receptor phosphorylation or stimulate downstream intracellular signaling pathways. VEGF<sub>165b</sub> competitively inhibits VEGF<sub>165</sub>-mediated proangiogenic activity, which results in the inhibition of endothelial cell proliferation and migration in vitro and neovascularization in different experimental models. The amino acid structure of VEGF<sub>165b</sub> is 95% to 96% identical to that of VEGF<sub>165</sub>. To date, the majority of studies that investigated VEGF expression in healthy and diseased tissues could not distinguish between the proangiogenic and antiangiogenic VEGF isoforms.

In 2004, Woolard et al showed that a monoclonal antibody raised against the terminal 9-amino acid sequence of human VEGF<sub>165b</sub> could specifically detect antiangiogenic VEGF<sub>165b</sub> but not proangiogenic VEGF<sub>165</sub>. By contrast, there is no commercially available antibody that specifically detects only the VEGF<sub>165b</sub> isoform. With the VEGF<sub>165b</sub>-specific antibody, it could be demonstrated that VEGF<sub>165b</sub> is widely expressed in most healthy human tissues, as well as in
human plasma, with levels consistent with known circulating levels of VEGF.\textsuperscript{10,11} VEGF\textsubscript{165b} has been reported to be downregulated in all cancers investigated thus far, as well as in other angiogenic conditions such as proliferative diabetic retinopathy and Denys-Drash syndrome.\textsuperscript{10–14,16–18}

Several previous studies reported an increased expression of VEGF in SSc skin and circulation but could not distinguish between the VEGF\textsubscript{165} and VEGF\textsubscript{165b} isoforms.\textsuperscript{6,21–25} In the present study, we used VEGF\textsubscript{165b}-specific primer pairs and antibodies and were able to show that antiangiogenic VEGF\textsubscript{165b} is selectively upregulated at both the mRNA and protein levels in the affected skin of SSc patients. Moreover, we provided evidence that circulating levels of VEGF\textsubscript{165b} are raised in SSc.

Our data also indicate that almost all VEGF detected in SSc skin and circulation is the antiangiogenic VEGF\textsubscript{165b} and that increased levels of VEGF\textsubscript{165b} are early and persistent features of the disease. In addition, we found that dermal MVECs isolated from SSc skin constitutively overexpress and release high levels of VEGF\textsubscript{165b} in the culture supernatant.

Consistent with previous reports,\textsuperscript{6,21} we confirmed the upregulation of VEGFR-2 on endothelial cells in SSc skin, a property that is also retained in cultured SSc MVECs. This might be interpreted as a frustrated attempt to provide effective angiogenesis through proangiogenic VEGF\textsubscript{165}/VEGFR-2 signaling enhancement in endothelial cells. However, in SSc, upregulated antiangiogenic VEGF\textsubscript{165b} may bind to endothelial VEGFR-2 with the same affinity as VEGF\textsubscript{165},\textsuperscript{11} thus preventing VEGFR-2 phosphorylation and downstream signaling and ultimately resulting in an insufficient angiogenic response to chronic tissue ischemia. Indeed, here we have shown that VEGFR-2 phosphorylation, activation of ERK1/2 MAPK, and capillary morphogenesis are impaired in SSc MVECs. We also found that in healthy MVECs, VEGFR-2 phosphorylation, downstream ERK1/2 activation, and angiogenesis may be significantly inhibited by treatment with recombinant VEGF\textsubscript{165b} or SSc-MVEC–conditioned medium that contains elevated levels of VEGF\textsubscript{165b}. Interestingly, the addition of anti-VEGF\textsubscript{165b} blocking antibodies to SSc-MVEC–conditioned medium not only prevented these inhibitory effects but even resulted in the promotion of healthy MVEC angiogenesis. Moreover, we also show that although severely impaired, SSc MVEC angiogenesis can be ameliorated by treatment with the combination of high-dose proangiogenic VEGF\textsubscript{165} and anti-VEGF\textsubscript{165b} neutralizing antibodies. Taken together, these data indicate that the uncontrolled expression of VEGF\textsubscript{165b} may have an important role in the defective angiogenesis found in SSc. It will be of

Figure 7. Upregulated VEGF\textsubscript{165b} correlates with increased expression of SRp55 in SSc skin. A, Representative immunostaining for VEGF\textsubscript{165b} (green) and SRp55 (red) in SSc and control skin. Upper panels, Weak expression of VEGF\textsubscript{165b} and SRp55 was observed in epidermis (e), dermal fibroblasts (arrow), and endothelial cells (arrowhead) in control skin. Middle and lower panels, Keratinocytes (e), fibroblasts (arrows), endothelial cells (arrowheads), and perivascular infiltrating inflammatory cells (asterisks) were strongly immunopositive for VEGF\textsubscript{165b} and SRp55 in SSc skin. Original magnification, ×63. Insets, High-magnification view of capillary endothelial cells (arrowhead) and fibroblasts (arrow) coexpressing VEGF\textsubscript{165b} and SRp55. Original magnification, ×100. Right panels, Scatterplots showing the distribution of sampled pixels plotted as a function of the red (y axis) and green (x axis) emission intensity; colocalized pixels in the image are included in the yellow-orange region. B, Total protein extracts of SSc and control skin were probed with anti-SRp55 antibodies. Results of a representative blot are shown. SSc 1 to 3 indicates early SSc skin; SSc 4 and 5, late SSc skin. Data of densitometric analysis of blot bands are represented as mean ± SD of optical density in arbitrary units (a.u.). *P<0.001 vs control.
interest to further explore whether signaling of VEGF<sub>165b</sub> through VEGFR-1 or neuropilin-1 may also be involved in SSc vasculopathy.

Finally, we also found in SSc skin a marked overexpression of the profibrotic TGF-β1 cytokine and SRp55 splicing factor that was strikingly correlated with increased levels of VEGF<sub>165b</sub> in different cell types. Cultured SSc MVECs also appeared to constitutively overexpress SRp55. Furthermore, we observed that stimulation of MVECs with TGF-β1 resulted in the upregulation of SRp55 and VEGF<sub>165b</sub>. Both TGF-β1 and SRp55 have previously been reported to favor distal splice-site selection in the exon 8 region of VEGF pre-RNA, leading to the upregulation of VEGF<sub>165b</sub> in vitro.<sup>10,19</sup> In the case of SRp55, this appears to occur through direct binding to the VEGF pre-RNA.<sup>19</sup> Conditional overexpression of SRp55 was shown to upregulate VEGF<sub>165b</sub>
relative to the VEGF<sub>165</sub> isoform, whereas SRp55 knockdown reduced expression of VEGF<sub>165b</sub>.<sup>19</sup> In SSc, elevated expression of TGF-β1 and SRp55 may therefore contribute to the switch from proangiogenic to antiangiogenic VEGF isoforms. TGF-β1 is known to be a key cytokine that drives the fibrotic process of SSc.<sup>1,2</sup> Therefore, the present results may also identify a novel link between 2 of the major aspects of SSc pathogenesis, ie, progressive fibrosis and vascular damage with lack of compensatory angiogenesis. Translational studies on preclinical animal models of SSc will further investigate the role of VEGF alternative splicing in SSc-related peripheral vascular disease.

In conclusion, the present data provide new insights into the pathogenesis of defective angiogenesis and vascular repair in SSc. Administration of proangiogenic VEGF<sub>165</sub> and other strategies aimed to counteract the constitutive upregulation of antiangiogenic VEGF<sub>165b</sub> or molecular regulation of VEGF pre-RNA splicing might represent potential therapeutic approaches to promote effective angiogenesis and capillary regeneration in SSc.

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Disclosures

None.

References


Novelty and Significance

What Is Known?

- Systemic sclerosis (SSc, or scleroderma) is a chronic, multisystem connective tissue disorder characterized by widespread microangiopathy, fibrosis, and autoimmunity that affects the skin and internal organs. Severe peripheral vascular complications may occur during the course of SSc.
- Although in SSc there is a lack of sufficient angiogenic response to chronic tissue ischemia that culminates in the loss of capillary vessels, the expression of the potent proangiogenic mediator vascular endothelial growth factor A (VEGF) has paradoxically been shown to be upregulated in this disease.
- Previous studies in the field did not distinguish between the proangiogenic VEGF165 and antiangiogenic VEGF165b isoforms that are generated by alternative splicing in the terminal exon of VEGF pre-RNA.

What New Information Does This Article Contribute?

- We show that the endogenous antiangiogenic VEGF165b splice variant is selectively upregulated in the skin and circulation of patients with SSc and that this property is retained in cultured dermal microvascular endothelial cells (MVECs) isolated from patients’ skin.
- We provide evidence that profibrotic transforming growth factor-β1 (TGF-β1) and serine/arginine protein 55 (SRp55) splicing factor may contribute to the switch from proangiogenic to antiangiogenic VEGF isoforms and that constitutive overexpression of antiangiogenic VEGF165b leads to impaired VEGF receptor-2 phosphorylation and reduced downstream signaling, thus preventing SSc MVECs from entering a suitable angiogenic program in vitro.
- Our in vitro findings also suggest that neutralization of elevated VEGF165b might represent a potential strategy to promote an effective angiogenic response in patients with SSc.

VEGF is considered one of the major proangiogenic regulators and is found to be overexpressed in most physiological and pathological angiogenic states. Although SSc is a nonangiogenic pathological condition characterized by progressive loss of the microvasculature, a number of studies have reported increased expression of VEGF in these patients. However, recent evidence has demonstrated that the VEGF primary transcript can be alternatively spliced in terminal exon 8, producing mRNA splice variants that are translated to the proangiogenic VEGF165 and antiangiogenic VEGF165b isoforms. Because the amino acid structure of VEGF165b is 95% to 96% identical to that of VEGF165, all previous studies in the field of SSc investigated VEGF expression using methodologies that could not distinguish between the proangiogenic and antiangiogenic VEGF isoforms.

The present study was designed to determine whether VEGF isoform expression could be altered in the skin and circulation of patients with SSc. Here, we show that VEGF165b is selectively overexpressed in SSc, and we highlight the molecular mechanisms by which this antiangiogenic VEGF isoform may play a crucial role in the defective angiogenesis and vascular repair process that characterizes SSc. Our results suggest that the combination of proangiogenic VEGF165 administration and VEGF165b neutralization might represent a potential therapeutic strategy to promote effective angiogenesis and capillary regeneration in SSc.
Overexpression of VEGF<sub>165b</sub>, an Inhibitory Splice Variant of Vascular Endothelial Growth Factor, Leads to Insufficient Angiogenesis in Patients With Systemic Sclerosis

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Overexpression of VEGF\(_{165b}\), an Inhibitory Splice Variant of Vascular Endothelial Growth Factor, Leads to Insufficient Angiogenesis in Patients With Systemic Sclerosis

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**METHODS**

**Patients, controls, skin biopsies and plasma samples**

Full-thickness skin biopsies were obtained from the clinically involved skin of one-third of the distal forearm of 35 patients with SSc (31 women, 4 men; mean ± SD age 48 ± 19 years) recruited from the Division of Rheumatology, Department of Biomedicine, University of Florence. The characteristics of the patients used for collection of forearm skin biopsies are listed in Online Table I. All patients fulfilled the criteria for SSc as suggested by LeRoy and Medsger.\(^1\) Patients were classified as having limited cutaneous SSc (lcSSc; n = 15) or diffuse cutaneous SSc (dcSSc; n = 20) subsets according to the criteria proposed by LeRoy et al.\(^2\) Disease duration was calculated since the first non-Raynaud’s symptom of SSc. Patients were further classified as being in the early (n = 21) or late (n = 14) phase of SSc according to disease duration (early lcSSc, disease duration <5 years; early dcSSc, disease duration <3 years)\(^3\) and to the clinical and pathological stage of skin involvement. We considered clinically involved skin for values of skin thickness ≥ 2, according to the modified Rodnan skin thickness score.\(^4,5\) In SSc, the skin score evaluates the thickness of skin as assessed by clinical palpation of 17 body areas on a scale of 0–3 (0 = normal, 1 = mild thickening, 2 = moderate thickening, 3 = severe thickening), and from the sum of the scores from all body areas, with a maximum possible total score of 51.\(^5\) All patients with SSc underwent a 15-day treatment washout before skin biopsy was performed. During this period only proton-pump inhibitors were allowed. Patients who could not undergo washout due to severe organ complications were not enrolled in the
Skin samples from the same forearm region of 23 age- and sex-matched healthy donors were used as controls (21 women, 2 men; mean ± SD age 46 ± 17 years). Each skin biopsy was divided into two specimens and processed for immunohistochemistry and biomolecular analysis, respectively. For immunohistochemistry, the specimens were fixed in 10% buffered formalin, dehydrated in graded alcohol series and embedded in paraffin. For RNA and protein extraction, skin specimens were immediately immersed in liquid nitrogen and stored at −80°C until use.

Blood samples from 61 patients with SSc (36 lcSSc and 25 dcSSc; 28 early and 33 late SSc phase; 57 women, 4 men; mean ± SD age 63.6 ± 11.5 years) and 30 age- and sex-matched healthy controls (28 women, 2 men; mean ± SD age 61.7 ± 12.2 years) were drawn, centrifuged (1,500 g, 15 minutes), and plasma was collected and stored in aliquots at −80°C until use. The characteristics of the patients used for collection of plasma samples are listed in Online Table II. Patients were not taking angiotensin converting enzyme inhibitors, corticosteroids, methotrexate, cyclophosphamide, D-penicillamine, iloprost, or other disease modifying drugs. Before blood sampling, they were washed out for 10 days from oral vasodilating drugs and for 2 months from intravenous alprostadil alphacyclodextrin.

All patients with SSc and control subjects signed an informed consent form, and the study complied with the principles of the Declaration of Helsinki and was approved by the local ethics committee.

**Isolation, culture, and stimulation of dermal microvascular endothelial cells (MVECs)**

Dermal MVECs were isolated from biopsy samples of involved skin of the forearm from 6 patients with dcSSc and from 6 healthy subjects. None of the patients were treated with immunosuppressive or other potentially disease-modifying drugs at the time of biopsy. To obtain the cells, skin biopsy samples were mechanically cleaned of epidermis and adipose tissue in order to obtain a pure specimen of vascularized dermis, and were treated as described elsewhere. Clusters of round-shaped cells were squeezed from microvessels and formed colonies composed of polygonal elements. Such colonies were detached with EDTA, and CD31-positive cells were subjected to immunomagnetic isolation with Dynabeads CD31 (Dynal ASA, Oslo, Norway). Isolated cells were further identified as MVECs by labeling with anti-factor VIII-related antigen and anti-CD105 (endoglin) antibodies, and by reprobing with anti-CD31 antibodies. Cells were maintained in complete MCDB medium (Sigma, St. Louis, MO, USA) supplemented with 30% heat-inactivated fetal bovine serum (FBS), 20 µg/ml endothelial cell growth supplement (ECGS; Calbiochem, Nottingham, UK), 10 µg/ml hydrocortisone, 15 UI/ml heparin, and antibiotics (100 UI/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml amphotericin). For stimulation experiments, MVECs were grown to 70% confluence, and then were washed three times with serum-free medium and serum-starved overnight in MCDB medium supplemented with 2% FBS. Medium was removed and cells were incubated with 2% FBS–MCDB medium containing recombinant human TGFβ1 (1 ng/ml; PeproTech, Rocky Hill, NJ, USA), VEGF₆₅ (10 ng/ml; R&D
Systems, Minneapolis, MN, USA), VEGF_{165}b (10 ng/ml; R&D Systems), or combination of VEGF_{165} and VEGF_{165}b (both 10 ng/ml) for 24 hours. In some experiments, after serum-starvation, healthy-MVECs were incubated with SSc-MVEC–conditioned medium in the presence or in the absence of 10 µg/ml of mouse monoclonal anti-human VEGF_{165}b blocking antibody or irrelevant isotype- and concentration-matched IgG (R&D Systems) for 24 hours. MVECs were used between the third and seventh passage in culture.

**Preparation of SSc MVEC–conditioned medium**

Confluent cultures of SSc-MVECs were washed twice with phosphate buffered saline (PBS) and incubated overnight in the presence of MCDB medium supplemented with 2% FBS. The culture supernatant was centrifuged at 1,500 rpm for 10 minutes, and either used immediately or stored at –20°C.

**RNA purification, cDNA synthesis, and semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was isolated from skin biopsy homogenates using the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen, Carlsbad, CA, USA). Renal cell carcinoma samples were used as disease controls.\(^8\) Extraction was performed according to the protocol described by the manufacturer, and RNA was eluted with RNase-free water. To remove the remaining genomic DNA, total RNA was treated using the RNase-free DNase Set (Qiagen, Milan, Italy) for 30 minutes at room temperature. First strand cDNA was synthesized using the QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer’s protocol. cDNA was then amplified using intronspanning primers that detect human VEGF_{165}b only, even in the presence of 1,000x greater concentration of VEGF_{165} mRNA, as described elsewhere.\(^8,9\) The forward primer was complementary to exon 4 (5’-GAGATGAGCTTCTCAGCAGC-3’), and the reverse primer to exon 8b (5’-TTAAGCTTCTCGTCTCTGAGAGATCTGCA-3’).\(^9\) The amplification protocol was: initial denaturation at 94°C for 2 minutes, 35 cycles of 94°C for 30 seconds (denaturation), 63°C for 30 seconds (annealing), and 72°C for 1 minute (extension), with a final extension at 72°C for 5 minutes using a TC-512 thermocycler (Techne, Cambridge, UK). PCR products were run on 1% agarose gels containing ethidium bromide and visualized under ultraviolet transillumination. This reaction consistently resulted in one amplicon at ~220 bp (consistent with VEGF_{165}b).\(^9\) A reverse primer complementary to exon 8a (5’-TCACCGCCTCGGCTTGTCACAT-3’) was also used that detects VEGF_{165} but not VEGF_{165}b.\(^9\) To normalize for equal amounts of total RNA, the transcript levels of β-actin (forward primer, 5’-GGACTTTCGAGCAAGAGATGG-3’; reverse primer, 5’-AGCACTGTGTGTTGGCGTGACAT-3’; amplicon size 234 bp) were assayed in the same samples.
Quantitative real-time RT-PCR

For mRNA quantification, after cDNA synthesis, SYBR Green real-time PCR was performed using the Applied Biosystems StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with melting curve analysis. The oligonucleotide primer pairs used were: VEGF_{165b} forward primer 5’-GAGATGAGCTTCCTACAGCAC-3’ and VEGF_{165b} reverse primer 5’-TTAAGCTTTTCAGCTTTCTGAGTGTACAC-3’ (annealing temperature 63°C, amplicon size 220 bp); VEGFR-2 forward primer 5’-ATGGGAACCGGAACCTCACTAT-3’ and VEGFR-2 reverse primer 5’-TCTTTTCTGGGCACCTTCTAT-3’ (annealing temperature 56°C, amplicon size 132 bp). The PCR mixture contained 1 µl of cDNA or distilled H$_2$O (for negative control), 0.5 µM of forward and reverse primers, 10 µl of 2x QuantiTect SYBR Green PCR Master Mix containing SYBR Green I dye, ROX passive reference dye, HotStarTaq DNA Polymerase, dNTP mix and MgCl$_2$ (Qiagen). Amplification was performed according to a standard protocol recommended by the manufacturer. Unspecific signals caused by primer dimers or genomic DNA were excluded by dissociation curve analysis, non-template controls and samples without enzyme in the reverse transcription step. 18S ribosomal RNA (forward primer, 5’-CGGCTACCACATCCAAGGAA-3’; reverse primer, 5’-GCTGGAATTACCGCGGCTGC-3’; amplicon size 187 bp) was measured as an endogenous control to normalize for the amounts of loaded cDNA. Differences were calculated with the threshold cycle (Ct) and comparative Ct method for relative quantification. All measurements were performed in triplicate.

Antibodies

The expression of VEGF$_{165b}$ was examined by immunohistochemistry and Western blotting using a mouse monoclonal IgG1 antibody that recognizes an epitope within a 9 amino acid sequence at the C-terminus of human VEGF$_{165b}$. It is affinity-purified against the human VEGF$_{165b}$ antigen from conditioned media of hybridoma cells, and is commercially available from R&D Systems (clone 56-1, cat. no. MAB3045) or Abcam (Cambridge, UK; clone MRVL56/1, cat. no. ab14994). This antibody has been shown using Western blotting and immunohistochemistry to specifically detect the VEGF$_{165b}$ isoform of VEGF, but not the pro-angiogenic VEGF$_{165}$ isoform, even when present at 10,000-fold greater concentrations. Protein samples extracted from various human tissues, when probed with this antibody, revealed immunoblotting bands of the same size as that detected using a pan-VEGF antibody. Three commercially available anti-VEGF-A antibodies were used to investigate the expression of all isoforms of VEGF (including VEGF$_{165b}$) – termed pan-VEGF: rabbit anti-human VEGF antibody A-20 and mouse anti-human VEGF antibody C-1 (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA; cat. no. sc-152 and sc-7269, respectively), and mouse anti-human VEGF antibody (BD PharMingen, Heidelberg, Germany; cat. no. 555036). These antibodies are raised to amino acid sequences of human VEGF protein which are identical in VEGF$_{165}$ and VEGF$_{165b}$ isoforms. For VEGFR-2/Flk-1, a rabbit polyclonal antibody was used (Abcam; cat. no.
Rabbit polyclonal anti-human SRp55 was from LifeSpan BioSciences (Seattle, WA, USA; cat. no. LS-B3092), and rabbit polyclonal anti-human pan-endothelial cell marker CD31 (cat. no. ab28364) and anti-human TGFβ1 (cat. no. ab74525) were from Abcam. Rabbit polyclonal anti-phospho-Akt (cat. no. ab38449), anti-Akt (cat. no. ab8805), anti-phospho-p44/p42 MAPK (phospho-ERK1/2) (cat. no. ab4819), and anti-p44/p42 MAPK (ERK1/2) (cat. no. ab17942) were purchased from Abcam. The anti-phosphotyrosine antibodies used in immunoprecipitation assays were from Sigma.

Histopathology, immunohistochemistry and confocal laser scanning microscopy

Paraffin sections (5 µm thick) were deparaffinized and either stained with hematoxylin and eosin for routine histology or processed for immunohistochemistry, as described elsewhere. For antigen retrieval, skin sections were boiled for 10 minutes in citrate buffer (10 mM, pH 6.0) followed by cooling of the slides for 20 minutes at room temperature in the same buffer. The sections were then washed 3 times in PBS and blocked for 1 hour at room temperature with 1% bovine serum albumin (BSA) in PBS (BSA/PBS). The slides were incubated overnight at 4°C with primary antibodies diluted in BSA/PBS (anti-VEGF<sub>165</sub>b, 2 µg/ml; anti-pan-VEGF A-20, 5 µg/ml; anti-pan-VEGF C-1, 1:100; anti-pan-VEGF from BD PharMingen, 1:100; anti-VEGFR-2, 1:50; anti-SRp55, 10 µg/ml; anti-CD31, 1:100; anti-TGFβ1, 1:25). After extensive washing in PBS, slides were incubated with secondary antibodies for 45 minutes at room temperature in the dark. The immune reactions were revealed using Alexa Fluor-488-conjugated goat anti-mouse IgG or Rhodamine Red-X-conjugated goat anti-rabbit IgG (1:200 dilution; Molecular Probes, Eugene, OR, USA) as secondary antibodies. Double immunostainings with mouse and rabbit reagents were performed by mixing primary antibodies and subsequently mixing fluorochrome-conjugated reagents. Irrelevant isotype- and concentration-matched IgG (Sigma) were used as negative controls. Cross-reactivity of secondary antibodies was tested in control experiments in which primary antibodies were omitted. Tissue sections were examined with a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany) equipped with a Leica PlanApo x63/1.40 NA oil immersion objective and a HeNe/Argon laser source for fluorescence measurements. Series of optical sections (1024x1024 pixels each) at intervals of 0.4 µm were obtained and superimposed to create a single composite image. Densitometric analysis of the intensity of immunofluorescent staining was performed on digitized images using the free-share ImageJ software (National Institutes of Health, Bethesda, MD, USA; online at http://rsbweb.nih.gov/ij).

Western blotting

Proteins were extracted from skin specimens by homogenization for 5 minutes in ice-cold lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.25% sodium dodecyl sulfate [SDS]) supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and the protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). For protein extraction
from cells, confluent monolayers of MVECs at basal condition or after specific treatments were
washed twice with PBS and then scraped in ice-cold lysis buffer. The solution was sonicated for 4
minutes, cleared by centrifugation for 30 minutes at 4°C at 15,000 rpm, and assayed for protein
content using Bradford’s method. Fifty micrograms of proteins were subjected to electrophoresis in
SDS-(10% or 12%) polyacrylamide gel under reducing conditions and then blotted to a nitrocellulose
transfer membrane (Amersham Biosciences, Piscataway, NJ, USA). In preliminary experiments,
recombinant human VEGF<sub>165</sub> and VEGF<sub>165b</sub> proteins (R&D Systems, cat. no. 293VE and 3045-VE,
respectively) were loaded, electrophoresed and blotted to test the specificity of anti-VEGF<sub>165b</sub>
and anti-pan-VEGF antibodies. The membranes were blocked in 5% nonfat dry milk with 0.05% Tween-
20 in PBS for 1 hour at room temperature, and then incubated overnight at 4°C with primary mouse or
rabbit anti-human antibodies (1:1,000 dilution). After washing and incubation with horseradish
peroxidase-conjugated anti-rabbit or anti-mouse IgG (Cell Signaling Technology, Danvers, MA, USA)
for 1 hour at room temperature, immune complexes were detected with the enhanced
chemiluminescence ECL detection system (Amersham Biosciences). The membranes were exposed to
autoradiographic films (Amersham Biosciences) for 1-30 minutes. Blots were stripped using Re-Blot
Plus Western Blot Recycling Kit (Chemicon International, Temecula, CA, USA) and reprobed with
rabbit monoclonal anti-human α-tubulin antibodies (1:1,000 dilution; Cell Signaling Technology) to
confirm similar loading of the gels and efficiency in electrophoretic transfer. Membranes probed with
anti-pAkt and anti-pERK1/2 antibodies were stripped and reprobed with anti-Akt (1:500 dilution) and
anti-ERK1/2 (1:1,000 dilution) antibodies, respectively. Densitometric analysis of the bands was
performed using ImageJ software.

**Immunoprecipitation**

The immunoprecipitations were carried out by using the Pierce Direct IP Kit (Thermo Fisher Scientific
Inc., Rockford, IL, USA). Immobilizing the antibody covalently to agarose beads, this method results
in purified antigen free from antibody contamination. To assess VEGFR-2 phosphorylation, anti-
phosphotyrosine antibody (Sigma) was coupled to beads, and protein samples extracted from MVECs
were incubated with the beads. The eluate was then subjected to electrophoresis and blotted with anti-
VEGFR-2 antibodies (Abcam).

**Enzyme-linked immunosorbent assay (ELISA)**

Plasma and cell culture supernatant levels of VEGF<sub>165b</sub> and pan-VEGF were measured by commercial
quantitative colorimetric sandwich ELISA from R&D Systems (cat. no. DY3045 and DVE00,
respectively) 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<sub>165b</sub> does not show crossreactivity to pro-angiogenic VEGF<sub>165</sub> isoform. The pan-VEGF ELISA
recognizes both VEGF\textsubscript{165} and VEGF\textsubscript{165}\textsubscript{b} isoforms. Optical density was measured by microtitre plate reader at 450 nm. Each sample was measured in duplicate.

**In vitro capillary morphogenesis assay**

In vitro capillary morphogenesis assay was performed in 96-well plates covered with Matrigel (BD Biosciences, Bedford, MA, USA). Matrigel (50 µl; 10–12 mg/ml) was pipetted into culture wells and polymerized for 30 minutes to 1 hour at 37°C, as described elsewhere.\textsuperscript{14} Healthy-MVECs (30 x 10\textsuperscript{3} cells/well) were incubated in MCDB medium containing 2% FBS, or in 2% FBS-MCDB medium supplemented with recombinant human VEGF\textsubscript{165} (10 ng/ml; R&D Systems), VEGF\textsubscript{165}\textsubscript{b} (10 ng/ml; R&D Systems), or combination of VEGF\textsubscript{165} and VEGF\textsubscript{165}\textsubscript{b} (both 10 ng/ml). In some experiments, healthy-MVECs were incubated with SSc-MVEC–conditioned medium in the presence or in the absence of 10 µg/ml of mouse monoclonal anti-human VEGF\textsubscript{165}\textsubscript{b} blocking antibody or irrelevant isotype- and concentration-matched IgG (R&D Systems). In other experiments, SSc-MVECs were plated on Matrigel-coated wells and incubated with their culture conditioned medium that in some points was supplemented with 10 ng/ml or 50 ng/ml of VEGF\textsubscript{165}, or with the combination of VEGF\textsubscript{165} (50 ng/ml) and anti-VEGF\textsubscript{165}\textsubscript{b} blocking antibodies (10 µg/ml). Irrelevant isotype-matched IgG were used to verify the specificity of the effect. Plates were photographed at 6 and 24 hours. Results were quantified at 24 hours by measuring the percent field occupancy of capillary projections, as determined by image analysis. Six to nine photographic fields from 3 plates were scanned for each point.

**Statistical analysis**

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) software for Windows, version 12.0 (SPSS, Chicago, IL, USA). Data are shown as mean ± SD, or median and range. The non-parametric Mann-Whitney U test for independent samples was used to test the probability of significant differences between groups. A value of p<0.05 was considered statistically significant.
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Online Figure I. Western blotting analysis of VEGF\textsubscript{165b} expression in protein homogenates from skin biopsies. Total protein extracts of skin samples were probed with anti-VEGF\textsubscript{165b}-specific antibodies. Treatment of skin protein homogenates with stronger reducing conditions yielded a single band of 23 kDa, which is consistent with the molecular weight of VEGF\textsubscript{165b} monomer. The preincubation of anti-VEGF\textsubscript{165b} primary antibody with excess of recombinant human (rh) VEGF\textsubscript{165b} protein abolished the blot staining. On the contrary, blot bands of 23 kDa could be still detected after probing with anti-VEGF\textsubscript{165b} primary antibody preincubated with excess of rh VEGF\textsubscript{165}. Results of a representative experiment are shown.
Online Figure II. Upregulated VEGF_{165}b correlates with increased expression of SRp55 in SSc skin. A, Representative microphotographs of double immunostaining for VEGF_{165}b (green) and SRp55 (red) in SSc and control skin. Upper panels, Weak expression of VEGF_{165}b and SRp55 is observed in epidermis, dermal fibroblasts and capillary vessels of control skin. Middle panels, Keratinocytes, fibroblasts, and endothelial cells are strongly double immunopositive for VEGF_{165}b and SRp55 in SSc skin. Lower panels, A large perivascular inflammatory infiltrate shows a strong double immunopositive signal for VEGF_{165}b and SRp55 in SSc hypodermis. Original magnification, ×63. B, Total protein extracts from SSc and control skin samples were probed with anti-SRp55 antibodies. Results of a representative blot are shown. SSc 6-8 = early SSc skin; SSc 9 = late SSc skin.
Online Figure III. Phosphorylation of ERK1/2 and Akt in in healthy-MVECs (H-MVEC) and SSc-MVECs (SSc-MVEC). Left panels, Total protein extracts from H-MVEC and SSc-MVEC were probed with anti-phospho-ERK1/2 (pERK1/2) and anti-phospho-Akt (pAkt) antibodies. Blots were then stripped and reprobed with anti-total-ERK1/2 and anti-total-Akt antibodies, respectively. SSc-MVEC show lower levels of pERK1/2 compared with healthy-MVECs, while no significant difference is observed in Akt phosphorylation. Right panels, Analysis of ERK1/2 and Akt phosphorylation in H-MVEC at basal condition and after stimulation with recombinant VEGF$_{165}$, VEGF$_{165b}$, combination of both, or SSc-MVEC-conditioned medium (c.m.) in absence or presence of anti-VEGF$_{165b}$ blocking antibodies. Treatment of H-MVEC with pro-angiogenic VEGF$_{165}$ results in a strong increase of pERK1/2, while incubation with VEGF$_{165b}$ alone or in combination with VEGF$_{165}$ inhibits ERK1/2 phosphorylation. SSc-MVEC c.m. reduces the levels of pERK1/2, whereas the addition of anti-VEGF$_{165b}$ blocking antibodies to SSc-MVEC c.m. results in increased pERK1/2 in H-MVEC. Treatment with VEGF$_{165b}$ or SSc-MVEC c.m. does not appear to inhibit phosphorylation of Akt in H-MVEC.
**Online Table I.** Demographic and clinical characteristics of the 35 patients with SSc used for collection of skin biopsies.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SSc patients (n = 35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years ± SD)</td>
<td>48 ± 19</td>
</tr>
<tr>
<td>Sex,</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>4 (11.4)</td>
</tr>
<tr>
<td>Female</td>
<td>31 (88.6)</td>
</tr>
<tr>
<td>Disease subset,*</td>
<td></td>
</tr>
<tr>
<td>lcSSc</td>
<td>15 (42.8)</td>
</tr>
<tr>
<td>dcSSc</td>
<td>20 (57.2)</td>
</tr>
<tr>
<td>Disease phase,**</td>
<td></td>
</tr>
<tr>
<td>Early SSc</td>
<td>21 (60.0)</td>
</tr>
<tr>
<td>Late SSc</td>
<td>14 (40.0)</td>
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<tr>
<td>Autoantibody positivity,</td>
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</tr>
<tr>
<td>ANA</td>
<td>34 (97.1)</td>
</tr>
<tr>
<td>Anti-Scl-70</td>
<td>16 (45.7)</td>
</tr>
<tr>
<td>ACA</td>
<td>13 (37.1)</td>
</tr>
<tr>
<td>Digital ulcers</td>
<td>21 (60.0)</td>
</tr>
<tr>
<td>Raynaud’s phenomenon</td>
<td>35 (100.0)</td>
</tr>
<tr>
<td>Skin score (mean ± SD)†</td>
<td>12.8 ± 7.5</td>
</tr>
<tr>
<td>Pulmonary fibrosis§</td>
<td>13 (37.1)</td>
</tr>
<tr>
<td>Pulmonary arterial hypertension*‡</td>
<td>4 (11.4)</td>
</tr>
</tbody>
</table>

Except where indicated otherwise, values are the number (%) of subjects.
*According to the criteria proposed by LeRoy et al.²
**Defined as: early lcSSc, disease duration <5 years; early dcSSc, disease duration <3 years.³ Disease duration was calculated since the first non-Raynaud’s symptom of SSc.
†Modified Rodnan skin thickness score.⁵
§Determined by high-resolution computed tomography scan.
*Confirmed by right heart catheterization.

ACA, anticentromere antibodies; ANA, antinuclear antibodies; Anti-Scl-70, anti-Scl-70 antibodies; dcSSc, diffuse cutaneous SSc; lcSSc, limited cutaneous SSc; SSc, systemic sclerosis.
### Online Table II. Demographic and clinical characteristics of the 61 patients with SSc used for collection of plasma samples.

<table>
<thead>
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<th>Characteristic</th>
<th>SSc patients (n = 61)</th>
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</thead>
<tbody>
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<td>Mean age (years ± SD)</td>
<td>63.6 ± 11.5</td>
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<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>4 (6.6)</td>
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<tr>
<td>Female</td>
<td>57 (93.4)</td>
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<tr>
<td>Disease subset,*</td>
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</tr>
<tr>
<td>lcSSc</td>
<td>36 (59.0)</td>
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<tr>
<td>dcSSc</td>
<td>25 (41.0)</td>
</tr>
<tr>
<td>Disease phase,**</td>
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</tr>
<tr>
<td>Early SSc</td>
<td>28 (45.9)</td>
</tr>
<tr>
<td>Late SSc</td>
<td>33 (54.1)</td>
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<tr>
<td>Autoantibody positivity,</td>
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<tr>
<td>ANA</td>
<td>58 (95.1)</td>
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<tr>
<td>Anti-Scl-70</td>
<td>21 (34.4)</td>
</tr>
<tr>
<td>ACA</td>
<td>32 (52.5)</td>
</tr>
<tr>
<td>Digital ulcers</td>
<td>28 (45.9)</td>
</tr>
<tr>
<td>Raynaud’s phenomenon</td>
<td>61 (100.0)</td>
</tr>
<tr>
<td>Skin score (mean ± SD)†</td>
<td>10.9 ± 6.5</td>
</tr>
<tr>
<td>Pulmonary fibrosis§</td>
<td>20 (32.8)</td>
</tr>
<tr>
<td>Pulmonary arterial hypertension*‡</td>
<td>7 (11.5)</td>
</tr>
</tbody>
</table>

Except where indicated otherwise, values are the number (%) of subjects.
*According to the criteria proposed by LeRoy et al.²
**Defined as: early lcSSc, disease duration <5 years; early dcSSc, disease duration <3 years.³ Disease duration was calculated since the first non-Raynaud’s symptom of SSc.
†Modified Rodnan skin thickness score.⁵
§Determined by high-resolution computed tomography scan.
*Confirmed by right heart catheterization.

ACA, anticientromere antibodies; ANA, antinuclear antibodies; Anti-Scl-70, anti-Scl-70 antibodies; dcSSc, diffuse cutaneous SSc; lcSSc, limited cutaneous SSc; SSc, systemic sclerosis.