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Bucillamine Induces the Synthesis of Vascular Endothelial Growth Factor Dose-Dependently in Systemic Sclerosis Fibroblasts via Nuclear Factor-κB and Simian Virus 40 Promoter Factor 1 Pathways

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ABSTRACT

The pathogenesis of systemic sclerosis (SSc) is characterized by activation of the immune system, impaired angiogenesis, and activated dermal fibroblasts. The effects of the immunosuppressive agent bucillamine (SA 96) on fibroblasts and angiogenic factors have not been examined. SA 96, and particularly its metabolite SA 981, increased the levels of vascular endothelial growth factor (VEGF) mRNA and protein dose-dependently in dermal fibroblasts from patients with SSc and healthy control subjects without influencing cell viability. SSc fibroblast cultures showed consistently a higher inducibility of VEGF than cultures from healthy control subjects. Preincubation with the SP-1 inhibitor mithramycin as well as blockade of nuclear factor (NF)-κB signaling with pyrrolidine dithiocarbamate treatment and IκB transfection reduced significantly the transcription of VEGF, indicating that both transcription factors contribute to the activation of VEGF by SA 981. Specific binding of NF-κB protein to its binding site after treatment with SA 981 was confirmed by electrophoretic mobility shift assay. In contrast, SA 981 did not influence the stability of VEGF mRNA as analyzed with actinomycin D assays. The study provides evidence for a role of NF-κB in the transcriptional regulation of the VEGF gene. SA 96 might have positive aspects on the impaired angiogenesis in patients with SSc.

Systemic sclerosis (SSc) is a fibrotic connective tissue disease that affects the skin and internal organs. Perivascular inflammatory infiltrates and a reduced capillary density characterize the early pathogenesis of SSc. These changes are often detectable before the excessive accumulation of extracellular matrix proteins occurs in later stages of the disease (Matucci-Cerinic et al., 1997). The reduced capillary density leads to a decreased blood flow with severe hypoxia in the skin of patients with SSc. Tissue hypoxia is usually a strong signal for the formation of new blood vessels from the pre-existing microvasculature, a process commonly known as

angiogenesis. However, despite the reduced blood flow and the reduced levels of pO_2, there is no evidence for a sufficient angiogenesis in the skin of patients with SSc (LeRoy, 1996).

The knowledge about the complex interactions that occur during angiogenesis has noticeably increased during the last few years (Distler et al., 2002b). Angiogenesis is a multistep process that is inhibited by angiostatic factors in the physiological situation. In a hypoxic environment and in inflammatory conditions such as rheumatoid arthritis, angiogenic growth factors are induced and outweigh their inhibitors. The overexpression of angiogenic growth factors results in an outgrowth of endothelial cells, which form a cord and migrate toward the most hypoxic areas. Finally, the migrated endothelial cell cord

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ABBREVIATIONS: SSc, systemic sclerosis; VEGF, vascular endothelial growth factor; SA 96, bucillamine (*N*-(2-mercapto-2-methylpropanoyl)-L-cysteine); SA 981, (4*R*)-7,7-dimethyl-6-oxo-tetrahydro-3H-1,2,5-dithiazepine-4-carboxylic acid; SA 679, N-[2-methyl-2(methylthio)propenacyl]-L-cysteine; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; Ct, threshold cycle; NF-κB, nuclear factor-κB; PDTC, pyrrolidine dithiocarbamate; HIF, hypoxia inducible factor; SP-1, simian virus 40 promoter factor 1; CMV, cytomegalovirus.

has to be stabilized by the recruitment of pericytes and smooth muscle cells (Distler et al., 2002b).

The vascular endothelial growth factor (VEGF) has been characterized as a key molecule in the induction of angiogenesis. VEGF binds to the tyrosine kinase receptors VEGF-R1 (flt-1) and VEGF-R2 (flk-1), which are specifically expressed on endothelial cells. VEGF is involved in several steps of angiogenesis, including vasodilation, proliferation and migration of endothelial cells as well as lumen formation (Distler et al., 2002b). During these processes, the concentration of VEGF is tightly regulated and critical to achieve its biological effects. Disruption of a single VEGF allele in mice, which results in a 50% reduction in the synthesis of VEGF, is lethal for the embryo because of severe defects in the vasculature (Carmeliet et al., 1996; Ferrara et al., 1996).

Bucillamine (SA 96) is a disease-modifying agent that is commonly used for the treatment of rheumatoid arthritis in Japan. SA 96 has structural similarities to D-penicillamine but contains two free sulfhydryl groups instead of one, which results in substantially different molecular and therapeutic effects compared with D-penicillamine. For example, SA 96 is able to suppress type II collagen-induced arthritis in rats and seems to be more efficient in the treatment of rheumatoid arthritis (Hayashi et al., 1991; Kim and Song, 1997). After administration, SA 96 is metabolized to the internal disulfide SA 981 and in a second pathway to the mono-S-methylated form SA 679 (Matsuno et al., 1998).

SA 96 has been characterized as an immunomodulatory agent with suppressive effects on various B and T cell functions (Hirohata and Lipsky, 1994), whereas the effects of SA 96 on the metabolism of stromal cells have not been addressed. Herein, we show that the internal disulfide SA 981, as well as SA 96 itself, is able to induce a dose-dependent increase of VEGF in cultured dermal fibroblasts. This induction was mediated by activation of NF-κB and SP-1 pathways but not by an increase in the stability of VEGF mRNA. Interestingly, SSc fibroblasts showed a significantly higher inducibility of VEGF by SA 981 and SA 96 than did cultured fibroblasts from healthy volunteers, indicating that SA 96 might have favorable effects on the reduced angiogenesis in SSc.

Materials and Methods

Patients and Fibroblast Cultures. Fibroblast cultures were obtained from skin biopsies of affected skin from patients with SSc at the University of Florence. All patients fulfilled the criteria for SSc suggested by LeRoy et al. (1988). Biopsies ($n = 6$) were taken from involved skin at the advancing edge of the indurated plaque. Control fibroblasts ($n = 5$) were obtained from skin biopsies of healthy age- and sex-matched volunteers. After enzymatic digestion of the skin biopsies with dispase II (Roche Diagnostics, Rotkreuz, Switzerland), fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS). Fibroblasts from passages 3 to 8 were used for the experiments. All patients signed a consent form approved by the institutional review board of the University of Florence.

Incubation with SA 96 and Metabolites. Fibroblasts grown to confluence in 12-well plates were used for all experiments. Twenty-four hours before the experiments, cell cultures were washed with PBS (Invitrogen, Basel, Switzerland) and the medium was changed. SA 96 and its metabolites SA 981 and SA 679 (kindly provided by Santen Pharmaceutical, Osaka, Japan) were dissolved in DMEM/ 10% FCS.

To determine the optimal dosage and incubation time for SA 96 and its metabolites, SSc fibroblast and normal control cultures were incubated for 6 to 40 h with different concentrations. Because these initial experiments suggested a dose-dependent effect on the levels of VEGF at doses between 0.05 and 1 mg/ml already after 6 h, these parameters were used for further analyses.

SA 96 and the metabolites SA 981 and SA 679 were added to the fibroblast cultures at final concentrations of 0.05, 0.15, 0.6, and 1 mg/ml. Cultures incubated with DMEM/10% FCS under the same conditions were used as control subjects. After incubation for 6 h at 37°C and 5% CO_2, cell-free supernatants were collected and stored at -20°C for further analysis by enzyme-linked immunosorbent assay (ELISA).

Trypan blue dye exclusion tests were carried out with treated as well as nontreated fibroblasts to examine the viability of the cells. The cells were incubated with 0.05% (w/v) trypan blue and the percentage of cells excluding dye was estimated using a Neubauer chamber. Each test was repeated at least three times. In addition, confluence and morphology of the cultured fibroblasts were assessed before and after incubation with SA 96 and its metabolites by light microscopy.

Quantitative Real-Time PCR. TRIzol LS Reagent (Invitrogen) was used for the isolation of total RNA. Total RNA was reverse-transcribed into cDNA with random hexamers and quantified for VEGF by single reporter real-time PCR (TaqMan) using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland). Samples without enzyme in the reverse transcription (non-RT samples) were used as a negative control for the real-time PCR. TaqMan probe and primer sequences for $VEGF_{121}$ (GenBank accession number X62568) were as follows: forward primer, 5'- ccc att gca gac caa aga aag-3'; reverse primer, 5'-cct gcc cgg ctc acc-3'; and TaqMan probe, 5'-agc aag aca aga aaa atg tga caa gcc ga-3'.

To normalize for the amounts of loaded cDNA, a predeveloped 18S assay (Applied Biosystems) was used as an endogenous control. After confirmation that the amplification efficiency of VEGF and the endogenous control 18S were approximately equal, differences were calculated with the threshold cycle (Ct) and the comparative Ct method for relative quantification. All measurements were performed in duplicates.

ELISA. Cell-free supernatants from stimulated and nonstimulated SSc and normal fibroblasts were analyzed for VEGF protein by quantitative sandwich ELISA according to the instructions of the manufacturer (R&D Systems, Wiesbaden, Germany). Concentrations were calculated using a standard curve generated with standards for VEGF provided by R&D Systems. The optical density was measured at 450 nm and corrected for subtract readings at 570 nm with the MRX microplate reader (Dynex Technologies, Chantilly, VA). The minimum detectable dose of VEGF with this assay is less than 5.0 pg/ml. All measurements were performed at least in duplicates.

mRNA Stability Assay. The half-life of VEGF mRNA in SA 981-treated cells and nonstimulated control subjects were determined by treating fibroblasts with actinomycin D (Sigma, Deisenhofen, Germany) as described by Ikeda et al. (1995). In brief, after 6 h of incubation with SA 981, actinomycin D at 10 μg/ml dissolved in dimethyl sulfoxide (Sigma) was added to the medium of SSc and normal fibroblasts to block transcription. After addition of actinomycin D, cells were returned to the same culture conditions as before. During the ensuing 8 h, fibroblasts were harvested every 2 h, total RNA was isolated, reverse transcribed, and analyzed with quantitative real-time PCR as described above.

Blockade of SP-1 Pathways with Mithramycin. Mithramycin binds to GC boxes and prevents sequential binding of SP-1, resulting in inhibition of the expression of several genes (Blume et al., 1991; Ryuto et al., 1996). To investigate the role of the SP-1 pathway in the stimulation of VEGF by SA 981, cell cultures were incubated with mithramycin (Sigma) at concentrations of 100 and 500 nM for a total

of 9 h. After 3 h, SA 981 was added with a final concentration of 1 mg/ml to the medium. To examine the effects of the SP-1 inhibitor mithramycin on the basal levels of VEGF, cultured fibroblasts were incubated with mithramycin without stimulation with SA 981. TRIzol LS reagent was used to isolate total RNA. After reverse transcription, the amounts of VEGF mRNA were quantified by Taq-Man analyses with 18S as an endogenous control.

Incubation with Pyrrolidine Dithiocarbamate (PDTC). To examine the role of the NF-κB pathway in the induction of VEGF by SA 981, the NF-κB inhibitor PDTC (Sigma) was dissolved in PBS at a final concentration of 400 μM (0.6572 mg/ml). After preincubation with PDTC for 1 h at 37°C and 5% CO_2, PDTC was removed and the cultured SSc and normal fibroblasts were stimulated with SA 981. Control experiments were performed by preincubation with PBS alone. To assess the effects of the NF-κB inhibitor PDTC on the constitutive synthesis of VEGF by cultured fibroblasts, the cells were preincubated with PDTC and treated as described without adding SA 981 to the supernatants.

Treatment with Cycloheximide. To assess, whether the induction of VEGF by SA 981 is a direct effect or requires the synthesis of other proteins, cultured SSc and normal dermal fibroblasts were preincubated with cycloheximide (Sigma), an inhibitor of protein synthesis, at a concentration of 20 μg/ml for 1 h as described previously (Varedi et al., 1997). After this time, the medium was removed and the cells were stimulated with SA 981 as described before. Again, quantification of VEGF mRNA was performed by single reporter real-time PCR normalized for 18S using the comparative Ct method.

Transfection with pCMV-IκBαM. For transfection, 2×10^5 fibroblasts were resuspended in 100 μl of Nucleofector Solution for human dermal fibroblasts (Amaxa, Cologne, Germany), which is adapted to stabilize this particular cell-type during nucleofection and creates the conditions needed for direct transfer of DNA into the nucleus during the electroporation (Varedi et al., 1997; Hamm et al., 2002). Afterward, 2 μg of IκBαM pCMV vector DNA or empty vector for control (BD Bioscience, Palo Alto, CA) were added, the mixture was transferred into an electroporation cuvette and placed in the Nucleofector device (Amaxa). Immediately after nucleofection, the cell suspension was transferred into six-well plates (OmniLab, Nett-mensletten, Switzerland) containing 1 ml of prewarmed DMEM. Medium was changed after 8 h. Positive control experiments with the pEGFP-C1 vector (BD Biosciences Clontech, Palo Alto, CA) and subsequent FACS analysis showed a strong protein synthesis in 60 ± 12% after 48 h. Therefore, the transfected fibroblasts were stimulated with SA 981 48 h after transfection as described above, RNA was isolated and the expression of VEGF was quantified with real-time PCR.

Electrophoretic Mobility Shift Assay. SSc and healthy fibroblasts were cultured to confluence in culture flasks (225 cm²) and incubated with SA 981 at a final concentration of 1 mg/ml or without SA 981 as a negative control. Cells were collected by scratching in ice-cold PBS 6 h after addition of SA 981. Nuclear extracts were prepared according to the protocol of Andrews and Faller (1991). The concentration of nuclear protein was determined with the bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL) to normalize for the amounts of protein within each experiment. Nonradioactive electrophoretic mobility shift assay was performed using an EMSA kit according to the manufacturer's instructions (Panomics Inc., Redwood City, CA). Three micrograms of nuclear protein were incubated for 30 min at 21°C with biotinylated oligonucleotides containing the NF-κB binding site (Panomics Inc., Redwood City, CA). As a negative control, the binding reaction was performed in the presence of an excess of unlabeled double-stranded oligonucleotide. The samples were electrophoretically separated (120 V, 1.5–2 h) in a nondenaturing polyacrylamide gel (6% with 2.5% glycerol) and blotted (300 mA, 30–40 min) on a Biodyne B (0.45 μm) positively charged nylon membrane (Pall, Basel, Switzerland). The transfer buffer contained 20% methanol, 0.27 M Tris, and 2 M glycine. After transfer,

the membrane was UV–cross-linked at 254 nm for 3 min using a Stratalinker UV cross-linker (Stratagene). The biotin was labeled with alkaline phosphatase-conjugated streptavidin (1:1000; DAKO, Glostrup, Denmark) and streptavidin was detected with CDP-Star substrate (Applied Biosystems) according to the manufacturer's instructions. Chemiluminescence signals were visualized by exposing the membrane to an Agfa Curix Ortho HT-A film (Agfa-Gevaert, Kontich, Belgium) for 2 min.

Western Blot Analysis. Untreated and SA 981-stimulated fibroblasts from patients with SSc and from healthy control subjects were washed three times with ice-cold PBS. After incubation on ice for 10 min, the homogenate was centrifuged at 1600g and 4°C for 5 min. Nuclear extraction buffer containing 20 mM HEPES (pH 7.5), 400 mM NaCl, 1 mM EDTA (pH 8.0), 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride was added to the pellet in a 1:1 ratio and incubated on ice for 15 min. After centrifugation (20,000g, 5 min, 4°C), supernatants were collected and stored at −80°C. The protein concentration of the lysates was measured using a bicinchoninic acid method (Pierce, Lausanne, Switzerland). Ten micrograms of nuclear protein extract were mixed with an equal volume of 2× electrophoresis sample buffer (100 mM Tris/HCl, pH 6.8, 10% SDS, 40% glycerol, 0.005% bromphenol blue, and 0.7 M 2-mercaptoethanol) and denatured by heating to 95°C for 10 min. Proteins were separated on 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Membranes were blocked in 5% nonfat dry milk with 0.05% Tween 20 in Tris-buffered saline, pH 7.4, for 1 h at room temperature before probing with monoclonal antibodies against HIF-1α for 90 min at room temperature (1:750; Novus Biologicals, Littleton, CO). After incubation with horseradish peroxidase-conjugated secondary antibodies (goat anti mouse-IgG, dilution 1:10000; Milan Analytica AG, La Roche, Switzerland) in 5% nonfat dry milk with 0.05% Tween 20 in Tris-buffered saline, pH 7.4, for 1 h at room temperature, bound antibodies were visualized using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Statistics. Data are expressed as mean ± S.E.M. The Wilcoxon signed rank test for related samples and the Mann-Whitney test for nonrelated samples were used for statistical analyses. A p value of less than 0.05 was considered statistical significant.

Results

Basal Synthesis of VEGF mRNA in Cultured Dermal Fibroblasts from Patients with SSc and from Healthy Control Subjects

VEGF mRNA was detectable in all fibroblast cultures by TaqMan analysis. Without treatment with SA 96 and its metabolites, there was no significant difference between fibroblasts from patients with SSc and those from healthy volunteers, with neither 10% nor 0.1% FCS (10% FCS: SSc fibroblasts, ΔCt 17.83 ± 0.40; normal fibroblasts, ΔCt 17.05 ± 0.71; 0.1% FCS: SSc fibroblasts, ΔCt 18.98 ± 0.40; normal fibroblasts, ΔCt 18.81 ± 0.61). Similarly, no significant differences were found in the levels of VEGF mRNA when the results were analyzed separately for diffuse and limited SSc (limited SSc, ΔCt 18.07 ± 0.41; diffuse SSc, ΔCt 17.59 ± 0.25).

SA 96 and Its Metabolites Induce VEGF mRNA in SSc Fibroblasts

To study the effects of SA 96 and its metabolites on the levels of VEGF, dermal fibroblast cultures from patients with SSc and from healthy control subjects were stimulated at different concentrations. Initial experiments showed that incubation for 6 h was sufficient to induce VEGF in cultured fibroblasts.

SA 981. After incubation with the internal disulfide SA 981, the highest levels of VEGF mRNA were measured. As illustrated in Fig. 1, A and B, there was a dose-dependent increase of VEGF mRNA in SSc fibroblasts that was as high as 11.1 ± 1.5 fold at 1 mg/ml of SA 981 compared with nonstimulated control subjects ($p < 0.05$). An increase could be seen in all SSc cultures (Fig. 1B). Interestingly, we found significant differences in the inducibility of SSc and normal fibroblasts by SA 981. Similar to SSc fibroblasts, SA 981 enhanced the levels of VEGF mRNA dose-dependently in normal fibroblast cultures (Fig. 1A). However, the levels of VEGF mRNA were consistently lower after incubation with SA 981 than in SSc fibroblast cultures (Fig. 1, A and C). This difference between SSc fibroblast cultures and normal fibroblast cultures reached statistical significance at a concentration of 1 mg/ml (normal, 5.2 ± 1.3; SSc, 11.1 ± 1.5 fold increase, $p < 0.05$; Fig. 1A).

SA 96. Similar to SA 981, SA 96 induced a dose-dependent increase of VEGF mRNA in SSc fibroblasts (Figs. 2, A and B). An induction of VEGF mRNA was again seen in all SSc cultures (Fig. 2B). However, the levels of VEGF mRNA were lower than after treatment with SA 981 (SA 96, 3.8 ± 0.2 fold increase; SA 981, 11.1 ± 1.5 fold increase). Normal fibroblast cultures were less inducible by SA 96 than SSc fibroblast cultures with a greater variance between individual cultures. No clear dose dependence was detectable in the levels of VEGF mRNA in normal fibroblasts (Fig. 2, A and C).

SA 679. The metabolite SA 679, in which one sulfhydryl group is *S*-methylated, showed effects on the levels of VEGF mRNA that were less clear than those of than SA 96 and the internal disulfide SA 981 (Fig. 3A). There was a significant

induction with SSc fibroblasts at a concentration of 1 mg/ml (SSc, 3.1 ± 0.5-fold increase compared with basal levels, $p < 0.05$), whereas at lower concentrations, no significant effects on the levels of VEGF mRNA were detected (Fig. 3, A and B). Healthy fibroblasts showed no significant induction of VEGF at any concentration of SA 679 (Fig. 3, A and C).

No differences were found between fibroblast cultures from patients with limited SSc and diffuse SSc after stimulation with SA 981, SA 96, and SA 679. As analyzed by the trypan blue dye exclusion test and cell morphology, SA 96 and its metabolites did not affect the viability of the cells (less than 5% trypan blue-positive cells in treated and nontreated cells). These data indicate that the effects on the levels of VEGF could not be ascribed to nonspecific cytotoxic effects.

SA 981 Induces VEGF on the Protein Level

Because the highest induction of VEGF mRNA was found with the internal disulfide SA 981 at a concentration of 1 mg/ml, supernatants from these experiments were used for ELISA. Interestingly, basal levels of VEGF protein without stimulation were higher in normal fibroblasts than in SSc fibroblasts when the cells were cultured with 10% FCS (SSc, 62 ± 47 pg/ml; normal, 140 ± 58 pg/ml; $p < 0.02$). These differences was also found under conditions with low growth factors (0.1% FCS), where VEGF protein was detectable only in two of five SSc cultures at low levels (14 ± 2 pg/ml), whereas VEGF was detectable in all normal fibroblast cultures in higher concentrations (92 ± 79 pg/ml). In agreement with the findings on the mRNA level, a significant increase of VEGF protein was seen in SSc fibroblast cultures after incubation with SA 981 compared with nonstimulated control
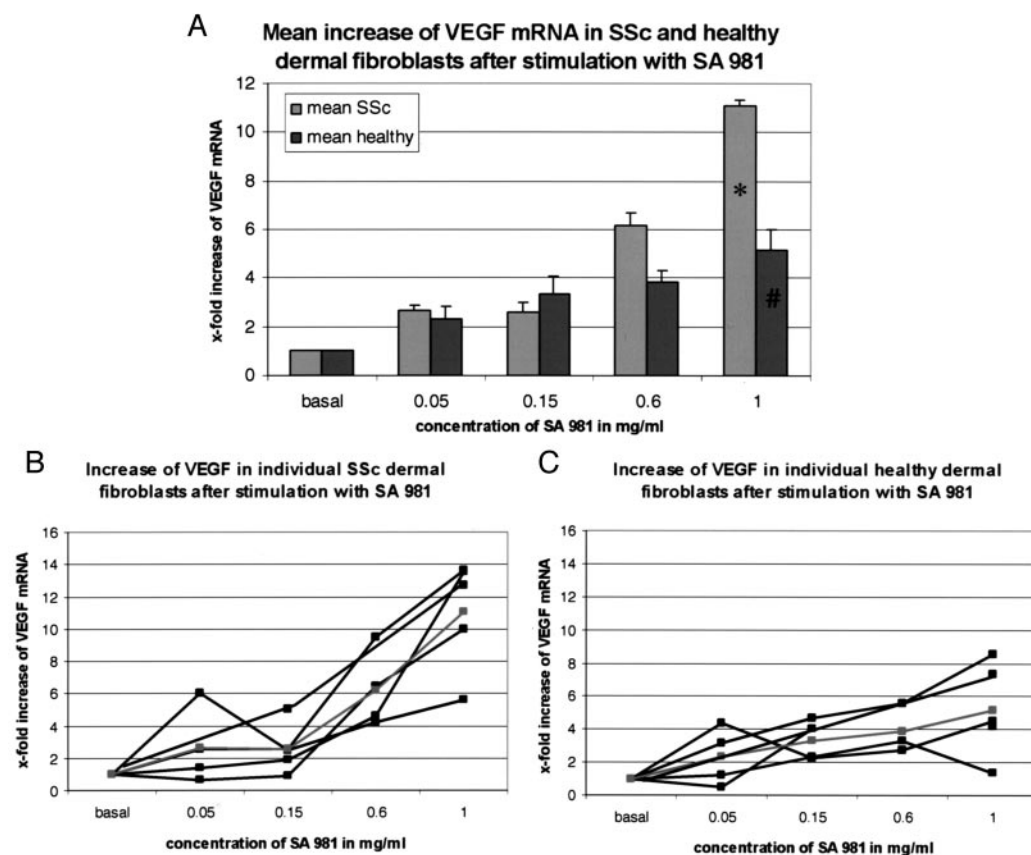

Fig. 1. Effects of SA 981 on the levels of VEGF mRNA. Dermal fibroblast cultures from patients with SSc and healthy control subjects were incubated with SA 981 and analyzed for VEGF mRNA by real-time PCR using the comparative threshold method. A, incubation with the internal disulfide SA 981 resulted in a dose-dependent increase of VEGF mRNA in both SSc (light gray columns) and healthy (dark gray columns) fibroblasts compared with basal levels (defined as 1). Healthy fibroblasts were significantly less inducible by SA 981 than SSc fibroblasts. B and C, synthesis of VEGF mRNA in individual SSc (B) and healthy (C) fibroblast cultures. Black lines indicate single fibroblast cultures from different patients/control subjects. Experiments were repeated at least three times for individual samples. Dark gray lines show mean fold increase of VEGF mRNA compared with basal levels. *, $p < 0.05$ compared with nonstimulated control subjects; #, $p < 0.05$ compared with -fold increase in SSc fibroblasts.
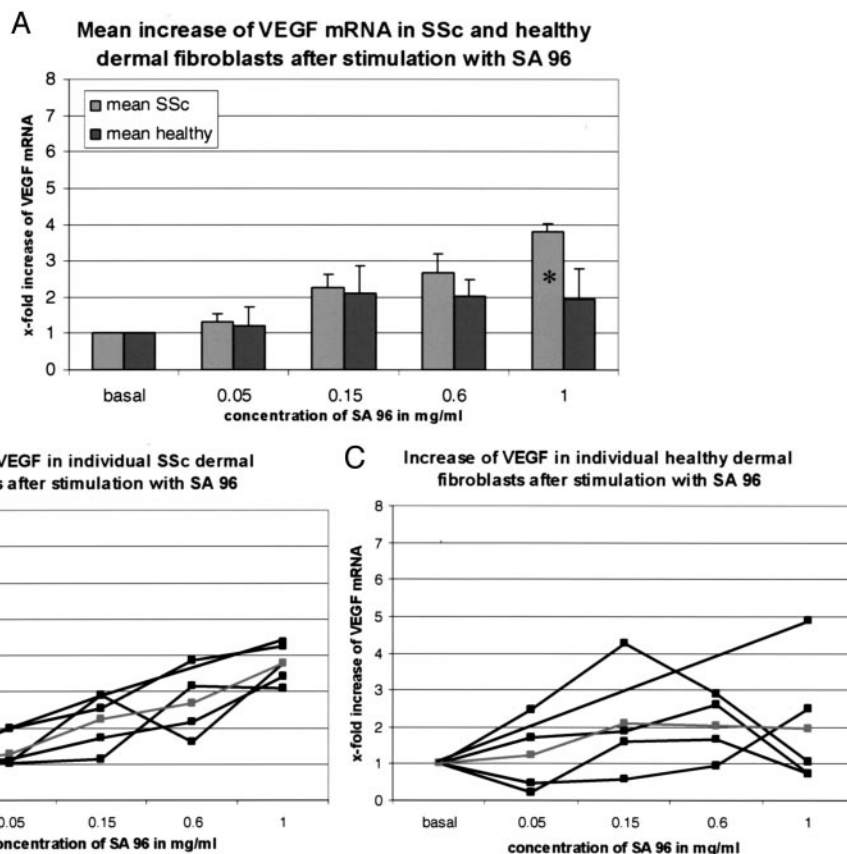
A

Mean increase of VEGF mRNA in SSc and healthy dermal fibroblasts after stimulation with SA 96

B

Increase of VEGF in individual SSc dermal fibroblasts after stimulation with SA 96

C

Increase of VEGF in individual healthy dermal fibroblasts after stimulation with SA 96

Fig. 2. Effects of SA 96 on the levels of VEGF mRNA. For definitions, see Fig. 1. A, similar to SA 981, SA 96 caused a dose-dependent increase of VEGF mRNA in SSc fibroblasts. However, levels of induction were lower with SA 96 than with SA 981. Again, healthy fibroblasts showed lower levels of VEGF mRNA than SSc fibroblasts and there was no obvious dose dependence after stimulation. B and C, synthesis of VEGF mRNA in individual SSc (B) and healthy (C) fibroblast cultures. Although VEGF was increased in all SSc cultures, some of the healthy cultures showed no increase of VEGF after stimulation with SA 96.
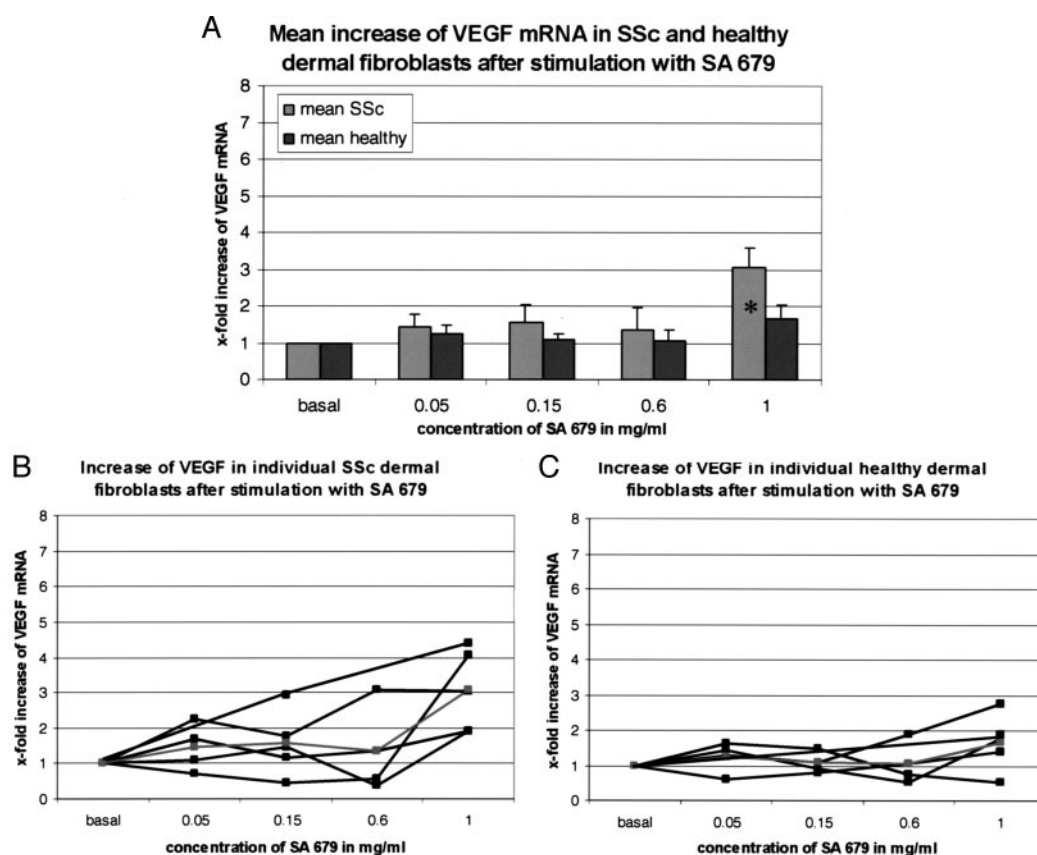
A

Mean increase of VEGF mRNA in SSc and healthy dermal fibroblasts after stimulation with SA 679

B

Increase of VEGF in individual SSc dermal fibroblasts after stimulation with SA 679

C

Increase of VEGF in individual healthy dermal fibroblasts after stimulation with SA 679

Fig. 3. Effects of SA 679 on the levels of VEGF mRNA. For definitions, see Fig. 1. A, in contrast to SA 981 and SA 96, SA 679 did not show a dose-dependent induction of VEGF mRNA. A significant increase was only seen after incubation with SA 679 at a concentration of 1 mg/ml in SSc fibroblast cultures. B and C, synthesis of VEGF mRNA in individual SSc (B) and healthy (C) fibroblast cultures. No significant effects were found in healthy fibroblasts. SSc fibroblasts showed an increase of VEGF in all cultures at a concentration of 1 mg/ml.

subjects (nonstimulated, 62 ± 47 pg/ml; stimulated, 169 ± 59 pg/ml; $p < 0.01$) (Fig. 4A). Whereas an induction of VEGF was seen in all SSc fibroblasts, the effects of SA 981 on healthy fibroblasts were more heterogeneous with no or minimal effects in individual cultures (Fig. 4B). In contrast to SSc fibroblasts and consistent with the mRNA analysis, the induction of VEGF by SA 981 did not reach statistical significance in healthy fibroblasts ($p > 0.05$). To test whether the induction of VEGF by SA 981 is a direct effect of SA 981, or whether this process requires the synthesis of other proteins as second mediators, the synthesis of new proteins was blocked with cycloheximide. Pretreatment of SSc and healthy dermal fibroblasts with cycloheximide did not reduce the induction of VEGF by SA 981.

Effects of SA 981 on the Stability of VEGF mRNA

It has been shown that hypoxia is able to induce VEGF as a result of both transcriptional activation and increased stability of mRNA (Ikeda et al., 1995). To investigate whether an altered stability of mRNA could be responsible for the increased levels of VEGF mRNA after incubation with SA 981, mRNA stability assays with actinomycin D were performed. As illustrated in Fig. 5, A and B, the levels of VEGF mRNA declined constantly after addition of actinomycin D in both normal and SSc fibroblasts. No significant differences in the stability of VEGF mRNA could be detected at any time point between SSc fibroblasts after treatment with SA 981 compared with nontreated SSc fibroblasts (Fig. 5A). Similarly, normal fibroblasts treated with SA 981 showed no differences in the stability of VEGF

mRNA compared with nontreated control subjects (Fig. 5B). Moreover, when the stability of VEGF mRNA was compared between SSc fibroblasts and normal fibroblasts under basal conditions (control subjects in Fig. 5, A and B), no significant differences were found. These data indicate that SA 981, in contrast to hypoxia, does not increase VEGF mRNA through an increase in the mRNA stability.

The Induction of VEGF upon Stimulation with SA 981 Is Not Mediated by HIF-1α

The 5'-flanking region of the VEGF gene contains functionally active HIF-1α binding sites and HIF-1α is one of the most potent stimuli of VEGF expression. To address whether the up-regulation of VEGF after stimulation with SA 981 is mediated by an induction of HIF-1α, Western blots for HIF-1α were performed. HeLa cells treated with cobalt chloride, a chemical stabilizer of HIF-1α were used as positive control subjects and showed an intense signal for HIF-1α. In contrast, HIF-1α protein was not detectable in SSc or healthy dermal fibroblasts, neither under basal conditions nor after stimulation with SA 981 (Fig. 6). This indicates that SA 981 does not stabilize HIF-1α and that the induction of VEGF HIF-1α by SA 96 and its metabolites is independent from HIF-1α.

SA 981 induces Transcription of VEGF mRNA via SP-1 and NF-κB Pathways

To search for signaling pathways that mediate the induction of VEGF by SA 981 in dermal fibroblasts, cultured cells were incubated with the SP-1 inhibitor mithramycin. Con-
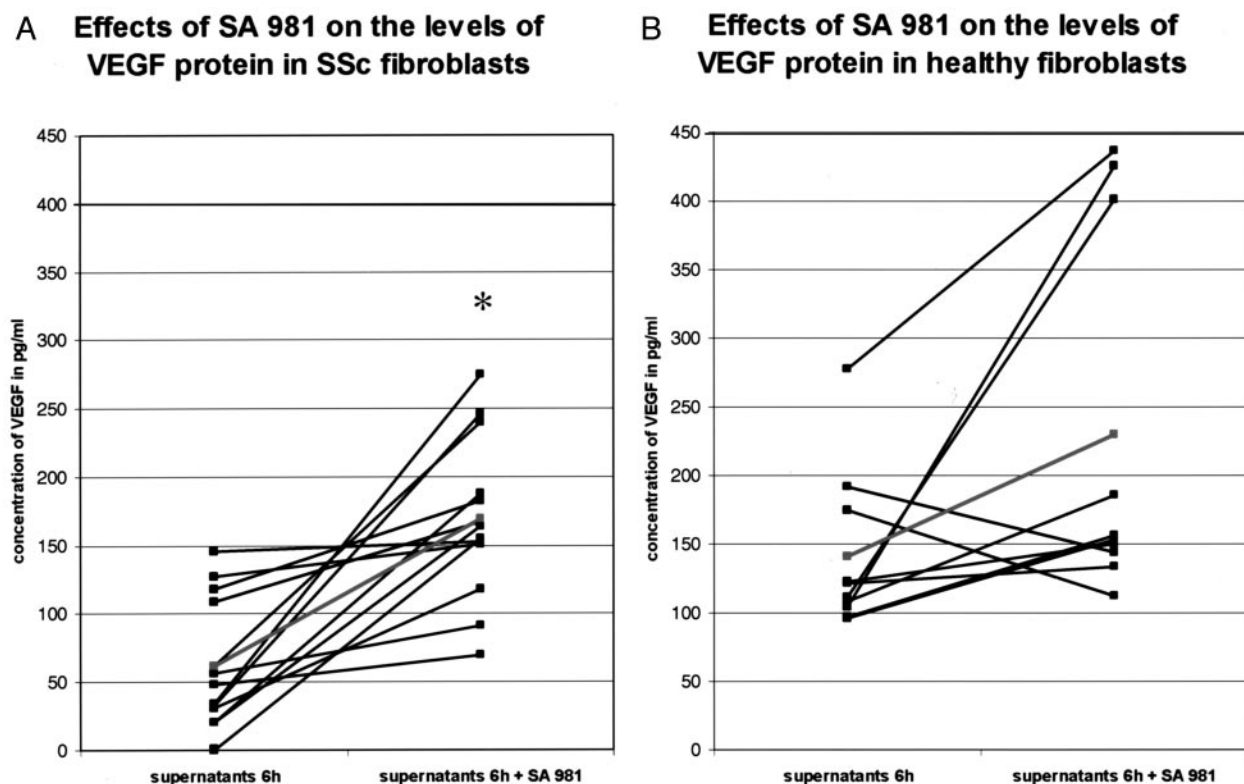

Fig. 4. Effects of SA 981 on the levels of VEGF protein in SSc and normal fibroblasts. Supernatants of fibroblasts incubated with the bucillamine metabolite SA 981 were analyzed for VEGF protein by ELISA. Results from individual cultures are shown. A, SA 981 induced an increase of VEGF protein in all SSc fibroblast cultures. The mean level of VEGF protein was 169 ± 59 pg/ml in stimulated fibroblasts compared with 62 ± 47 pg/ml in nonstimulated control subjects (dark gray line). B, basal levels of VEGF protein were higher in normal fibroblasts compared with SSc fibroblasts. In agreement with the mRNA results, normal fibroblasts showed a smaller increase of VEGF protein compared with SSc fibroblasts, which did not reach statistical significance. Dark gray line indicates mean levels of VEGF protein in the supernatants.

sistent with the results from earlier experiments, SA 981 induced VEGF in both SSc and normal fibroblasts and a significantly higher transcription of VEGF was observed in SSc fibroblasts compared with normal fibroblasts. In SSc fibroblasts, treatment with mithramycin decreased SA 981-induced VEGF mRNA significantly in a dose-dependent manner (Fig. 7 A, light gray columns: 100 nM mithramycin, $69 \pm 6\%$ reduction; 500 nM mithramycin, $89 \pm 8\%$ reduction; $p < 0.05$). When nontreated SSc fibroblasts were incubated with 500 nM mithramycin, the constitutive transcription of VEGF mRNA was also reduced (Fig. 7B). However, this reduction was significantly lower than the reduction after stimulation with SA 981 (SA 981, $89 \pm 8\%$ reduction; nontreated, $74 \pm 2\%$ reduction, $p < 0.05$). Levels of VEGF mRNA were still higher in SA 981-stimulated dermal fibroblasts than in nonstimulated control subjects after treatment with 500 nM mithramycin, indicating that maximal SP-1 inhibition was not achieved with this dose of mithramycin. These results suggest that the SP-1 pathway contributes to the constitutive synthesis of VEGF and is even more activated after treatment with SA 981.

Similar to SSc fibroblasts, mithramycin decreased SA 981-induced VEGF mRNA in normal fibroblasts significantly in a dose-dependent manner (Fig. 7A, dark gray columns: 100 nM mithramycin, $56 \pm 12\%$ reduction; 500 nM mithramycin, $96 \pm 5\%$ reduction; $p < 0.05$). This reduction was in the same range as observed in SSc fibroblasts. In addition, no differences were observed in the inhibition of the constitutive transcription of VEGF between healthy and SSc fibroblasts (healthy, $74\% \pm 2\%$ reduction; SSc, $75 \pm 2\%$ reduction). These data indicate that the differences between SSc and

normal fibroblasts in the levels of VEGF mRNA after incubation with SA 981 cannot be ascribed to differences in the activation of the SP-1 pathway. As assessed by the trypan blue dye exclusion test and cell morphology, mithramycin did not affect the viability of the cells.

To investigate whether SA 981 induces the transcriptional activation of VEGF through the NF-κB pathway, fibroblast cultures were incubated with the NF-κB inhibitor PDTC before stimulation with SA 981. In SSc fibroblasts, preincubation with PDTC reduced the levels of SA 981-induced VEGF mRNA by $88 \pm 10\%$ (Fig. 8 A, light gray columns; $p < 0.05$). To analyze the contribution of the NF-κB pathway to the basal transcription of VEGF mRNA in SSc fibroblasts, SSc fibroblasts were incubated with PDTC and handled as described before, but without stimulation with SA 981. Under these conditions, the levels of VEGF mRNA were reduced by $42 \pm 10\%$ (Fig. 8B, light gray columns, $p < 0.05$), indicating that NF-κB contributes to the constitutive synthesis of VEGF in SSc fibroblasts and is strongly activated after incubation wit SA 981.

Normal fibroblasts were again less inducible with SA 981 than SSc fibroblasts. Similar to SSc fibroblasts, pretreatment with PDTC reduced the levels of VEGF mRNA by $98 \pm 16\%$ (Fig. 8A, dark gray columns; $p < 0.05$), whereas basal levels were reduced by $58 \pm 10\%$ (Fig. 8B, dark gray columns; $p < 0.05$). These results suggest that the differences between SSc and normal fibroblasts in inducibility by SA 981 are not the result of a different activation of the NF-κB pathway in these cells. Similar to mithramycin, levels of VEGF mRNA were higher in SA-981 stimulated dermal fibroblasts than in nonstimulated control subjects after treatment with PDTC.
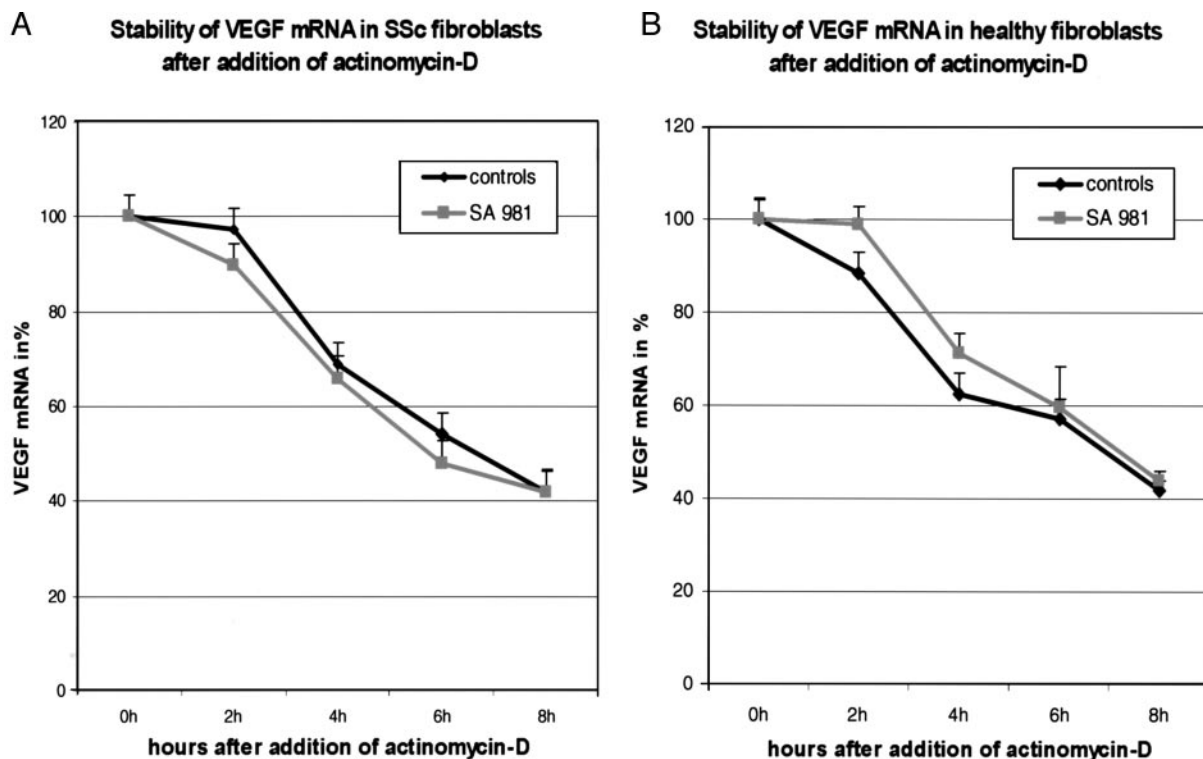
Fig. 5. Effects of SA 981 on the stability of VEGF mRNA. After stimulation with SA 981, mRNA stability assays with actinomycin D were performed. The amounts of VEGF mRNA were analyzed by real-time PCR every second hour after addition of actinomycin D. A, in SSc fibroblasts, no significant difference in the amounts of VEGF mRNA between treated and nontreated fibroblasts could be detected 2, 4, 6, and 8 h after addition of actinomycin D. B, similarly, normal fibroblasts treated with SA 981 showed no difference in the stability of VEGF mRNA compared with nontreated normal fibroblasts, indicating that the half-life of VEGF mRNA was not altered by SA 981.

To address the role of NF-κB signaling in the induction of VEGF by SA 981 in a more specific way, dermal fibroblasts were transfected with the pCMV-IκBαM expression vector and then stimulated with SA 981. This vector contains a mutated form of IκB that does not undergo stimulation-induced phosphorylation and thus remains bound to NF-κB. Treatment of mock-vector transfected fibroblasts with SA 981 resulted in a strong induction of VEGF mRNA and this up-regulation was again greater in SSc fibroblasts than in healthy control subjects. Consistent with the PDTC inhibitory data, the induction of VEGF was reduced by 49 ± 15% in IκB-transfected SSc fibroblasts compared with mock-transfected control subjects. Similar results were obtained with IκB transfected healthy fibroblasts, which showed a reduction by 56 ± 7%.

As a third independent method, EMSAs with labeled double-stranded oligonucleotide probes corresponding to binding sites for NF-κB were performed. After incubation with SA 981 in SSc fibroblasts, a strong signal for the NF-κB/probe complex was observed, whereas no signal was detected in nonstimulated SSc fibroblasts (Fig. 9). The specificity of the strong shift for NF-κB after stimulation was confirmed by the addition of an excess of unlabeled probe, which inhibited completely the formation of the NF-κB/probe complex. Similarly, healthy fibroblasts showed a NF-κB shift after incubation with SA 981 (Fig. 9). Unspecific signals were again excluded by unlabeled probe experiments.

Discussion

The results of the present study demonstrate novel effects of SA 96 on the metabolism of dermal fibroblasts showing a dose-dependent induction of the potent angiogenic factor VEGF after incubation with SA 96 and its metabolites. Thus, SA 96 might have favorable effects on vascular changes in SSc apart from the well-described actions in immune and inflammatory pathways. Vascular changes are a hallmark in the pathogenesis of early SSc and are characterized by a reduced capillary density and a concomitant lack of sufficient angiogenesis despite the presence of stimuli such as hypoxia and inflammatory cytokines (Matucci-Cerinic et al., 1997; Distler et al., 2002c). Along this line, we have recently shown that patients with SSc without fingertip ulcers have higher serum levels of VEGF than patients with fingertip ulcers. Whereas patients with SSc in general have higher VEGF levels than control patients, these data indicated that the levels of VEGF need to be shifted above an individual threshold level to be protective against ischemic manifestations such as fingertip ulcers (Distler et al., 2002a). Therefore, SA 96, which results in an increase of VEGF in particular in SSc
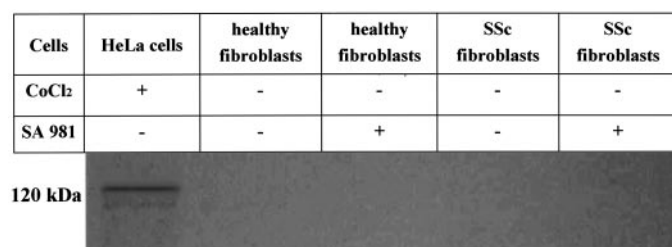
Cells	HeLa cells	healthy fibroblasts	healthy fibroblasts	SSc fibroblasts	SSc fibroblasts
CoCl₂	+	-	-	-	-
SA 981	-	-	+	-	+
120 kDa					

Fig. 6. Western blot for HIF-1α with nuclear extracts from SSc and healthy fibroblasts with and without SA 981 to address the role of HIF-1α in the induction of VEGF by SA 981. A strong signal for HIF-1α at 120kDa was observed in HeLa cells incubated with cobalt chloride, which were used as positive control subjects. However, HIF-1α protein was not detectable in either untreated or SA 981 stimulated cultured dermal fibroblasts.
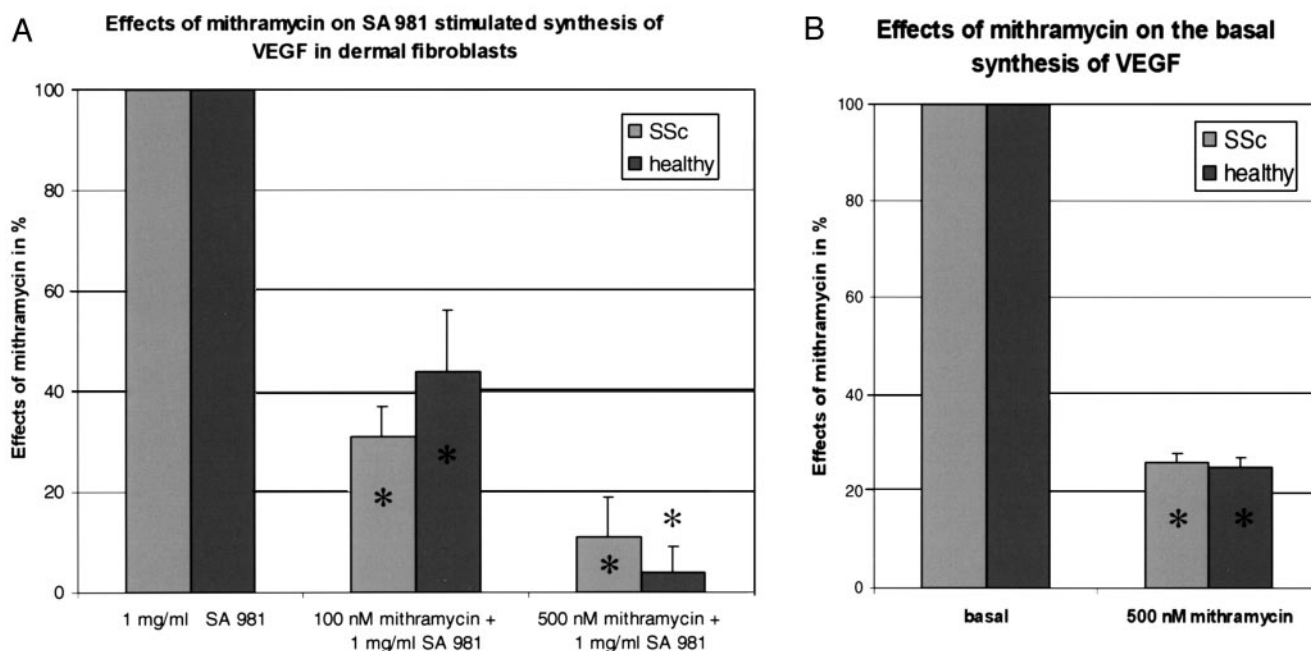

A Effects of mithramycin on SA 981 stimulated synthesis of VEGF in dermal fibroblasts

B Effects of mithramycin on the basal synthesis of VEGF

Fig. 7. Effects of the SP-1 inhibitor mithramycin on the induction of VEGF mRNA in skin fibroblasts treated with SA 981. SA 981 increased the transcription of VEGF in all SSc and normal cultures, with a significantly greater induction in SSc fibroblasts (see also Fig. 1, A–C). A, in SSc and healthy fibroblasts, mithramycin reduced the SA 981-induced transcription of VEGF significantly in a dose-dependent manner. No significant differences were found in the levels of reduction between SSc and healthy fibroblasts. B, the constitutive transcription of VEGF was also inhibited by treatment with mithramycin but to a lower extent than that seen in fibroblasts treated with SA-981. These results suggest that the SP-1 pathway contributes to the constitutive synthesis of VEGF and is even more activated after treatment with SA 981. *, $p < 0.05$ compared with fibroblasts without mithramycin.
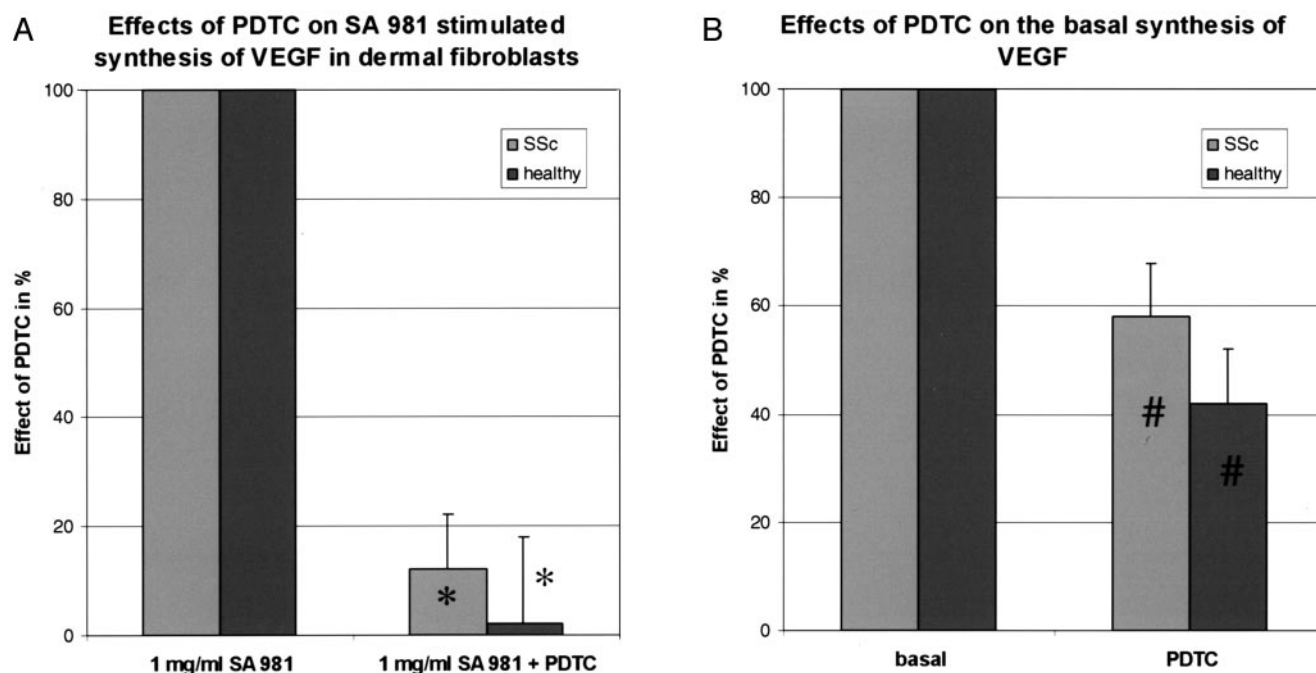
A. Effects of PDTC on SA 981 stimulated synthesis of VEGF in dermal fibroblasts

B. Effects of PDTC on the basal synthesis of VEGF

Fig. 8. Effects of the NF-κB inhibitor PDTC on the induction of VEGF mRNA in skin fibroblasts treated with SA 981. A, after pretreatment with PDTC, the effects of SA 981 on the levels of VEGF mRNA were reduced significantly in both SSc (light gray columns, $88 \pm 10\%$; $p < 0.05$) as well as normal fibroblasts (dark gray columns, $98 \pm 16\%$; $p < 0.05$). Differences in the level of reduction between SSc and healthy fibroblasts were not significant. B, basal levels of VEGF mRNA were decreased by $42 \pm 10\%$ in SSc fibroblasts (light gray columns; $p < 0.05$) and by $58 \pm 10\%$ in healthy fibroblasts (dark gray columns; $p < 0.05$). This reduction of basal levels was significantly lower than the reduction after stimulation with SA 981 (Fig. 7A). *, $p < 0.05$ compared with fibroblasts without PDTC. #, significant reduction of the basal sythesis of VEGF by PTDC, $p < 0.05$.

fibroblasts, could not only reduce the inflammatory component of SSc but also prevent the manifestation of ischemic symptoms.

The effects of SA 96/SA 981 on the synthesis of VEGF might be specific for certain tissues and cell types. In addition, there might be disease-specific reactions and/or dose-dependent differences in the various cell-types. In synovial fibroblasts from patients with rheumatoid arthritis, in whom the induction of VEGF and the initiation of angiogenesis as well as the induction of certain proinflammatory cytokines through NF-κB would be an unwanted event, Nagashima et al. (1999) found no effects or even a decrease of lipopolysaccharide-induced production of VEGF after treatment with SA 96 and SA 981, suggesting contrary effects of SA 981 on VEGF synthesis in synovial fibroblasts. Similarly, at lower doses, SA 96 has been reported to inhibit VEGF in bovine retinal microcapillary endothelial cells (Koyama et al., 2002). Interestingly, dermal fibroblasts from healthy control subjects showed a consistently smaller induction of VEGF after treatment with SA 981 than those from patients with SSc. These results indicate that the high induction of VEGF by SA 981 might be specific for SSc, although the molecular mechanisms that account for those differences remain to be identified.

The intracellular signaling pathways of SA 96 and its metabolites in different cell types have not been addressed in detail. SA 96 and the mono S-methylated metabolite SA 679, which both contain at least one free sulfhydryl group, required the presence of $CuSO_4$ to exert their inhibitory effect on the mitogen induced proliferation and IL-2 production of T cells (Hashimoto and Lipsky, 1993). The addition of the peroxide scavenger catalase reversed these effects, indicating that the inhibition of T cell functions by SA 96 and SA 679

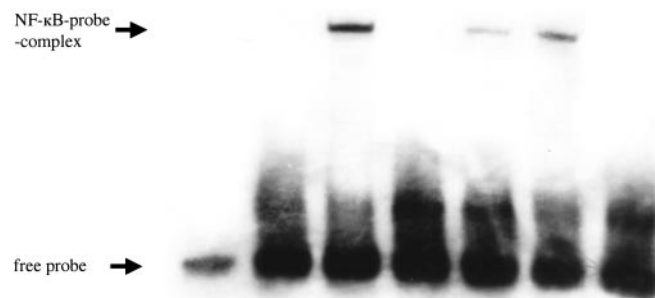| Cell type	Free probe	SSc	SSc	SSc	healthy	healthy	healthy
SA 981	-	-	+	+	-	+	+
Cold probe	-	-	-	+	-	-	+

Fig. 9. EMSA with nuclear extracts to examine the activation of NF-κB in SSc and healthy fibroblasts after stimulation with SA 981. Lane 1, free probe without nuclear extracts; lane 2, nuclear extract from nonstimulated SSc fibroblasts with NF-κB-probe; lane 3, nuclear extract from SSc fibroblasts stimulated with SA 981 with NF-κB-probe; lane 4, nuclear extract from SSc fibroblasts stimulated with SA 981 with NF-κB-probe and unlabeled NF-κB-probe; lane 5, nuclear extract from nonstimulated healthy fibroblasts with NF-κB-probe; lane 6, nuclear extract from healthy fibroblasts stimulated with SA 981 with NF-κB-probe; lane 7, nuclear extract from healthy fibroblasts stimulated with SA 981 with NF-κB-probe and unlabeled NF-κB-probe. Strong signals for NF-κB-DNA complexes in lanes 3 and 6 demonstrate an activation of the transcription factor NF-κB in SSc and normal skin fibroblasts after stimulation with SA 981. In contrast, there was only weak basal activation in nonstimulated cells (lanes 2 and 5). The specificity of these signals for NF-κB-DNA complexes was proven by incubating NF-κB-DNA-probes and nuclear extracts from stimulated SSc and normal fibroblasts with an excess of unlabeled unlabeled NF-κB probe. As shown in lanes 4 and 7, the signal of the specific complex disappeared completely, indicating that this signal was not caused by unspecific binding.

depend on the production of hydrogen peroxide after oxidation of the free thiol groups catalyzed by copper ions (Mita and Matsunaga, 1990; Hashimoto and Lipsky, 1993). Interestingly, hydrogen peroxide has been shown to stimulate the expression and DNA binding activity of NF-κB (Schreck et al., 1991), which was also activated in our study.

The intramolecular disulfide SA 981 does not contain a free sulfhydryl group. Along this line, SA 981 showed inhibitory effects on T cell functions independently from the presence of $CuSO_4$ as well as from the formation of hydrogen peroxide (Hashimoto and Lipsky, 1993). Because SA 981 exerted effects on the synthesis of VEGF in the present study, which were even stronger than those seen with SA 96, intracellular signaling pathways of SA 981 might be different from SA 96. Notably, SA 981 is a hydrophobic compound and can most probably enter cells rapidly (Hashimoto and Lipsky, 1993).

Hypoxia induces VEGF mRNA via both an increase in transcriptional activation and a prolonged half-life of VEGF mRNA (Finkenzeller et al., 1995; Ikeda et al., 1995). Similar mechanisms have been found for other hypoxia-regulated genes, such as tyrosine hydroxylase, but do not account for the induction of VEGF by cytokines (Ryuto et al., 1996). As indicated by the mRNA stability assay, SA 981 does not affect the half-life of the VEGF mRNA and probably regulates the levels of VEGF mRNA via transcriptional activation. In addition, treatment with SA 981 does not lead to an accumulation and translocation of HIF-1α protein, suggesting HIF-1α–independent mechanisms for the induction of VEGF by SA 981.

Our results from the inhibition experiments indicate that the intracellular signaling pathways of SA 981 in dermal fibroblasts might include the activation of SP-1. Transcription of VEGF can be induced by a variety of environmental stimuli and intracellular signaling cascades. The promoter of VEGF contains a cluster of SP-1 binding sites in its proximal region but lacks a typical TATA box in vicinity of the transcription start site (Fig. 10). The transcription of TATA less promoters in turn can be activated by SP-1, which often binds to clusters of SP-1 binding sites near these promoters (Ryuto et al., 1996). In fact, deletion mutants of the VEGF promoter, as well as point mutations of SP-1 binding sites, indicated that the proximal SP-1 cluster of the VEGF promoter is critical for the regulation of VEGF transcription in response to a variety of stimuli (Ryuto et al., 1996; Finkenzeller et al., 1997; Mukhopadhyay et al., 1997). As shown in Fig. 9, additional SP-1 binding sites exist upstream of the proximal SP-1 cluster that also seem to contribute to the regulation of VEGF transcription in certain conditions (Salimath et al., 2000). Notably, it can not be excluded from our experiments that binding of other transcription factors with GC-rich binding sequences are responsible for the effects seen with the Sp-1 inhibitor mithramycin. Mithramycin is not absolutely specific for Sp-1 and might also prevent binding of factors such as Egr-1, which frequently has binding sites that overlap with those of Sp-1.

The results from experiments with the NF-κB inhibitor PDTC, transfection with IκB, a specific cellular antagonist of NF-κB signaling and with the EMSA for NF-κB show, on multiple experimental levels, that NF-κB in addition to SP-1 mediates the induction of VEGF by SA 981. These results are of particular interest, because the transcriptional regulation of VEGF by NF-κB has been a matter of discussion during the last years. The 5′-flanking region of the VEGF promoter has no typical NF-κB binding motif (Yoshida et al., 1997). However, transfection of human prostate cancer cells with the NF-κB inhibitor IκBαM was associated with decreased activity of a luciferase reporter, which was under control of a 5′-flanking region and 3′-untranslated region of the VEGF gene (Huang et al., 2001). Moreover, similar to the induction by SA 981, signaling via the NF-κB pathway has been suggested recently to play a role in the transcriptional regulation of the VEGF gene in human ovarian cancer cells (Huang et al., 2000). In addition, administration of NF-κB antisense oligonucleotides inhibited partially the TNF-α- and platelet-activating factor–dependent production of VEGF in human vascular endothelial cells (Yoshida et al., 1997; Ko et al., 2002). Consistent with our results, these observations suggest that there might be NF-κB–like binding sites in the 5′- and/or 3′ regulatory regions of the VEGF gene. Interestingly, as analyzed with the MatInspector software, a NF-κB-like binding site exists at position 1713 to 1727 (−650 to −636) of the VEGF promoter sequence (Fig. 10).

In conclusion, our study shows for the first time that SA 96 and its metabolites increase the synthesis of the potent angiogenic growth factor VEGF dose-dependently in dermal fibroblasts and might thereby induce angiogenesis in the SSc skin. In addition, our study provides indicates that NF-κB can regulate the transcription of VEGF, possibly via NF-κB–like binding sites in the VEGF promoter.
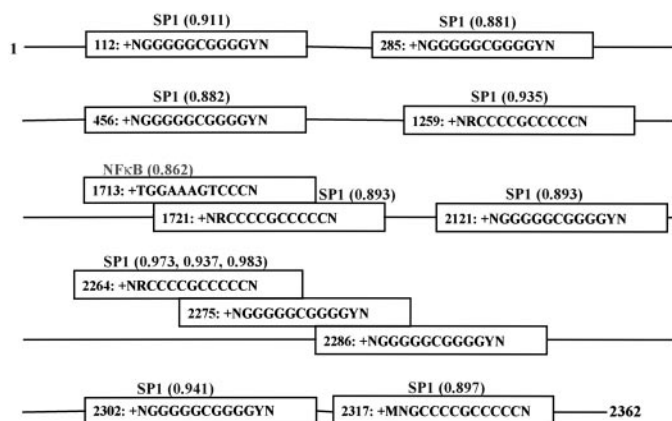
Fig. 10. Human VEGF promoter with NF-κB and SP-1 binding sites as predicted by MatInspector (GenBank accession number AF095785). Numbers in parentheses indicate the matrix similarity. Only matrix similarities higher then 0.80 are shown.

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