Inhibition of angiogenesis in lipodermatosclerosis: Implication for venous ulcer formation

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Abstract. Lipodermatosclerosis refers to skin induration of the lower extremities characterized by tortous, hyperpermeable vessels preceding venous leg ulcerations. Protein ligands and receptor tyrosine kinases that specifically regulate endothelial cell function are mainly involved in physiological as well as in disease-related angiogenesis. These ligand/receptor systems include the vascular endothelial growth factor (VEGF) and the angiopoietin (Ang) families and their receptor the tyrosine kinase with immunoglobulin-like domains (Tie-2) as well as the VEGF receptor family (VEGF-R1 and VEGF-R2). In the present study, the contribution of these endothelium-specific ligand/ receptor systems in tissue samples of lipodermatosclerosis was evaluated. Our results provide evidence, that the mRNA-transcripts of VEGF ($p<0.01$), Ang-1 ($p<0.1$), Ang-2 ($p<0.1$) and VEGF-R1 ($p<0.01$) were significantly upregulated in all samples of lipodermatosclerosis in comparison with healthy skin by using reverse transcriptase-polymerase chain reaction. On protein level VEGF ($p<0.01$), Ang-1 ($p<0.1$), Ang-2 ($p<0.1$) and VEGF-R1 ($p<0.01$) were significantly elevated as well. Solely for Tie-2 and for VEGF-R2 no statistical difference could be detected on mRNA and protein level in patients with lipodermatosclerosis in comparison with healthy skin. By immunohistochemistry we confirmed upregulated protein expression for VEGF, Ang-1, Ang-2 and VEGF-R1 compared with healthy skin. Our findings strongly suggest that an imbalance between these ligand/receptor systems might contribute to the pathophysiology of advanced stages of chronic venous insufficiency. Inhibition of angiogenesis could significantly impact the tissue breakdown in lipodermatosclerosis and could hereby enable the formation of venous leg ulcerations.

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Abbreviations: Ang, angiopoietin; VEGF, vascular endothelial growth factor; VEGF-R, vascular endothelial growth factor receptor; Tie-2, tyrosine kinase with immunoglobulin-like domains 2

Key words: angiogenesis, lipodermatosclerosis, vascular endothelial growth factor, angiopoietin, VEGF receptor

Introduction

Lipodermatosclerosis is associated with venous circulatory disorders of the lower extremities preceding chronic venous ulceration. The scleroderma-like hardening of lipodermatosclerosis is characterized by avascularization and anti-proliferation of dermal microvascular endothelial cells with perivascular fibrin cuffs surrounding dermal capillary vessels and loss of papillary structures (1). The stage preceding venous leg ulcers represents lipodermatosclerosis of the lower limb and is very common in the Western world. Previous studies have demonstrated that angiogenesis is inhibited in venous leg ulcers and many of these ulcers have been noted to show avascular and fibrous areas as well.

Angiogenesis is implicated in the pathogenesis of a variety of disorders such as proliferative retinopathies, rheumatoid arthritis, different tumors and wound healing (2-6). Tightly regulated signalling pathways control the directed proliferation, sprouting and migration of endothelial cells that occur during vasculogenesis and angiogenesis. Evidence accumulating over the last decade has established the fundamental role of vascular endothelial growth factor (VEGF) as a key regulator of normal and abnormal angiogenesis. It had been suggested that microcirculatory alterations in venous disease could be associated to increased concentration of VEGF. Vascular endothelial growth factor is a chemotactic agent and a potent endothelial cell mitogen which influences the vascular permeability (7,8). The biological effects of VEGF are mediated by two high-affinity tyrosine kinase

Table I. Primer sequences used for polymerase chain reaction.

Angiogenic agent	Sense (5'-3')	Antisense (5'-3')
VEGF	CCAGCACATAGGAGAGATGAGCTTC	CACCGCCTCGGCTTCTCACAT
Ang-1	GAGTTTATTTTTGCCATTACCAGTCAGAGG	CCCGCAGTATAGAACATTCCATTTAGATTG
Ang-2	CGTGAGGATGGCAGCGTTGAT	ATGGTTGTGGCCTTGAGCGAATAG
Tie-2	TGATTGACACTGGACATAACTTTG	ACGTGCTGGTCTTCATTCTTG
VEGF-R1	GTCATTCCCTGCCGGGTTACGTC	CGATGTTTCACAGTGATGAATGCT
VEGF-R2	GGCAGCTCACAGTCCTAGAGCGT	GGCCCGCTTAACGGTCCGTAG
ß-microglobulin	CCTGGAGGCTATCCAGCGTA	GTTCACACGGCAGGCATACT

VEGF, vascular endothelial growth factor; Ang, angiopoietin; Tie-2, tyrosine kinase with immunoglobulin-like domains 2; VEGF-R, vascular endothelial growth factor receptor.

receptors, VEGF-receptor 1 (VEGF-R1) and VEGF-receptor 2 (VEGF-R2) (9,10). Recent data indicate that VEGF signalling through VEGFR-2 is the major angiogenic pathway, and blockage of VEGF/VEGF-R2 signalling is the first anti-angiogenic strategy. Vascular endothelial growth factor-receptor 2 (VEGF-R2) which is strongly selective for vascular endothelium enhances endothelial cell proliferation and migration, as well as tubule formation (8). In contrast VEGF-R1 seems to act as a negative regulator of VEGF-mediated angiogenesis and as a stimulator of pathological angiogenesis (11). Furthermore VEGF-R1 has been reported to enhance vascular permeability (12).

In addition, specific regulation of the endothelial cell function include angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) and their receptor Tie-2 belonging to immunoglobulin-like homology domains (Tie) family (13). Angiopoietins exert no mitogenic activity on vascular endothelium. Instead, Tie-2 and angiopoietin-1 are mainly involved in endothelial cell-smooth muscle cell communication and in generating maturity as well as integrity of vessels. Tie-2 activation promotes vessel assembly and maturation by mediating survival signals for endothelial cells and regulating the recruitment of mural cells. Ang-1 acts in a paracrine agonistic manner inducing Tie-2 phosphorylation and subsequent vessel stabilization. Ang-1 was shown to support endothelium integrity, whereas Ang-2 had the opposite effect and promoted blood vessel destabilization and regression in the absence of survival factors VEGF (14-17). Ang-2 primes the vascular endothelium to exogenous cytokines and induces vascular destabilization at higher concentrations. The characterization of Ang-1 and Ang-2 as agonist and antagonist is based on the ability of Ang-2 to bind the Tie-2 receptor and to inhibit the pro-angiogenic action of Ang-1.

Avascularization and antiproliferation of dermal micro-vascular endothelial cells seem to be features of lipodermatosclerosis. Previously, VEGF was proven to be elevated on transcriptional level in non-healing venous leg ulcers and was found to be elevated in wound fluid as well (18-20). Lauer *et al* suggested that VEGF could lose its inefficacy to support the wound healing process due to an increased proteolysis and degradation (21). Our study was designed to define whether the protein VEGF, the ligands Ang-1, Ang-2, the receptor Tie-2 and the VEGF receptor tyrosine kinases VEGF-R1, VEGF-R2 are altered on mRNA and on protein level in lesional skin of lipodermatosclerosis.

Materials and methods

Clinical specimens. Patients with lipodermatosclerosis have been characterized according to the international clinical CEAP-classification of chronic venous insufficiency (CVI). The diagnosis was confirmed clinically by duplex scanning, doppler sonography, and by photoplethysmography. Patients punch biopsies from lipodermatosclerosis extending to the subcutaneous fat of the lower portion of the leg. These sites were scored as extensive according to the CEAP-classification (LDS: clinical score = 2). Biopsy specimens were snap-frozen in liquid nitrogen and stored at -80˚C until further processing. Lipodermatosclerosis was proven by conventional histology. Control biopsies from healthy volunteers were taken from the lower portion of the leg. All subjects provided informed written consent, and procedures involving humans were approved by the Ethics Committee of the Health Authority.

Monoclonal antibodies. Monoclonal antibodies (mAb) (*mouse IgG*) against VEGF (7G7), Tie-2 (αTek9), VEGF-R1 (4C8-10), VEGF-R2 (19H7a) as well as the polyclonal serum detecting both angiopoietin-1 and -2 were from the Institute of Molecular Oncology, Tumor Biology Center, Freiburg (22).

Reverse transcriptase-polymerase chain reaction. The mRNA-expression of VEGF, Ang-1 and Ang-2, Tie-2, VEGF-R1 and VEGF-R2 was analysed semiquantitatively by reverse transcriptase-polymerase chain reaction (23). Briefly, total mRNA was prepared from tissue sections using an RNeasy kit (Qiagen, Hilden, Germany). RT-PCR was performed by converting 1 μg of total RNA to cDNA using random hexanucleotide primers followed by PCR amplification of the respective cDNA fragments. All oligonucleotides were designed to recognize a unique sequence exclusive for each cDNA. The control gene ß-microglobulin served as reference. Specific primers were designed for VEGF, Ang-1, Ang-2, Tie-2, VEGF-R1 and VEGF-R2 (Table I). Polymerase chain reaction was performed using a GeneAmp PCR termal cycler (Perkin-Elmer, Weiterstadt, Germany) and consisted

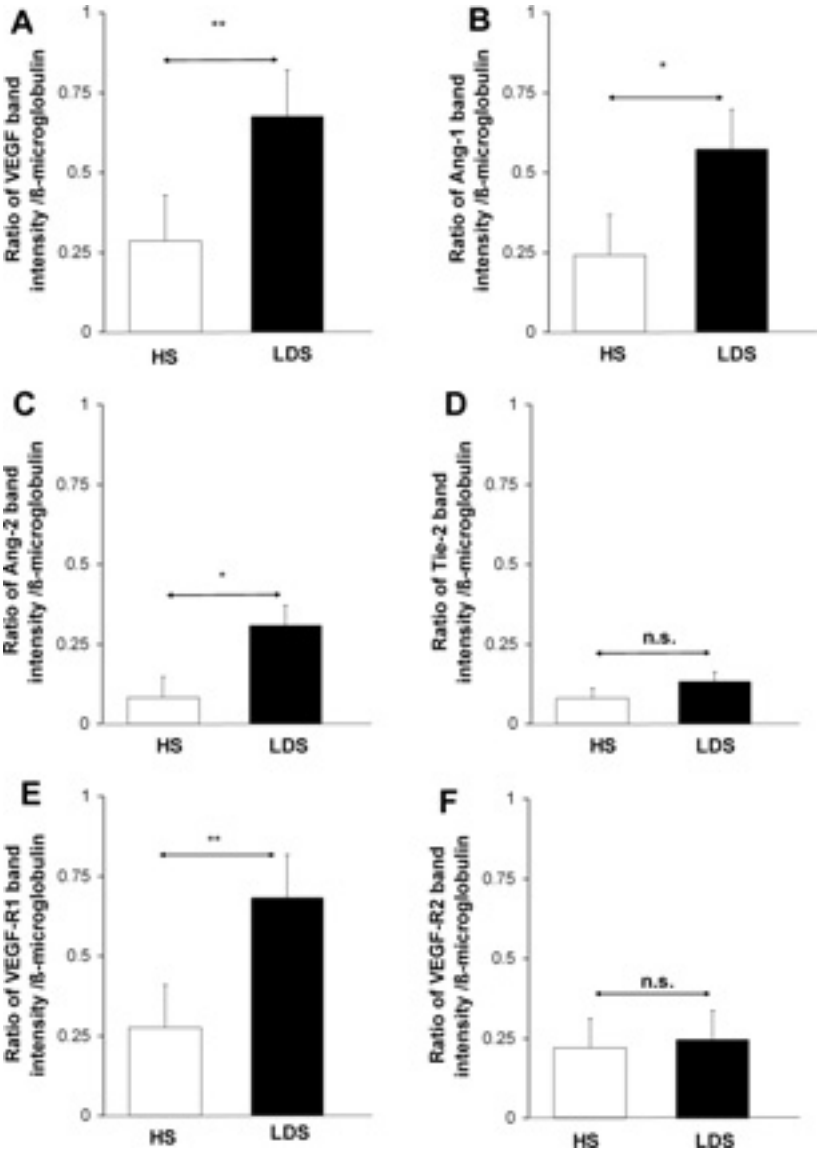

Figure 1. Gene expression for VEGF (A), Ang-1 (B), Ang-2 (C), Tie-2 (D), VEGF-R1 (E) and VEGF-R2 (F) on mRNA-level in patients with lipodermatosclerosis. Densitometric evaluation of generated mRNA-products shown as the ratio of relative intensity of band staining for VEGF, Ang-1, Ang-2, Tie-2, VEGF-R1 and VEGF-R2 in healthy controls (HS) (white columns) and lipodermatosclerosis (LDS) (black columns). Data are means ± SEM (n=6, for healthy skin; n=7, for leg ulcer group). The significance of difference was determined by an unpaired Student's t-test. Differences were considered significant at $^{*}p<0.1$; $^{**}p<0.01$ and not significant (n.s.).

of cycles of denaturation (94˚C, 1 min), ramped annealing (55˚C, 1 min) and extension (72˚C, 1 min). The generated products were subjected to electrophoresis on a 2% agarose gel and were visulized by ethidium bromide staining. To compare the mRNA expression of different biopsies, signals of VEGF, Ang-1, Ang-2, Tie-2, VEGF-R1 and VEGF-R2 were normalized to ß-microglobulin and the ratio was calculated. In order to assure linear cDNA amplification in our experiments, different amplifying cycles were checked (30-35 cycles).

Immunoblotting. Protein expression of VEGF, Ang-1, Ang-2, Tie-2, VEGF-R1 and VEGF-R2 were analysed by Western blot. Skin samples were pulverized and solubilized with a micro-dismembrator from Braun Biotech Int. (Melsungen, Germany). Samples with equal protein content were separated by 10% SDS-PAGE and electroblotted to nitrocellulose membrane. The proteins were visualized with the indicated primary and the ECL chemiluminescence system with peroxidase-conjugated secondary antibodies (Amersham, Braunschweig, Germany).

Immunohistochemistry. For light microscopic immunohistochemistry 5 μm cryostat sections were used to identify Ang-1 and Ang-2, Tie-2, VEGF-R1 and VEGF-R2. Sections were mounted on Superfrost Plus slides and allowed to dry overnight at room temperature. This was followed by a fixation in cold acetone for 10 min and a wash with PBS three times for 5 min each time. All samples were exposed to 3% H_2O_2 (DAKO LSAB 2 Kit, Carpinteria, USA) for 10 min to block endogenous peroxidases, washed 3 times with PBS, and subjected to a protein blocking solution for 15 min consisting of PBS with 2% serum albumin. Sections were stained with the appropriate primary antibody for 2 h at room

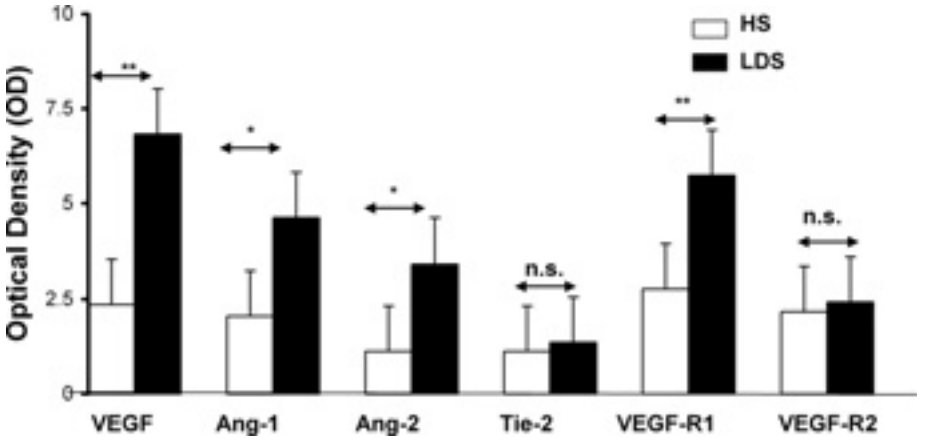

Figure 2. Protein expression of VEGF, Ang-1, Ang-2, Tie-2, VEGF-R1 and VEGF-R2 in extracts of lipodermatosclerosis was determined by immunoblotting. Densitometric evaluations of immunoblots VEGF, Ang-1, Ang-2, Tie-2, VEGF-R1 and VEGF-R2 from lipodermatosclerosis (LDS) (black columns) and healthy skin (HS) (white columns). Data are means ± SEM (n=10, for lipodermatosclerosis; n=10 for controls). The significance of difference was determined by an unpaired Student's t-test and is indicated in each mapped group.

temperature. Primary antibodies were provided by the Institute for tumor biology in Freiburg. The primary antibodies were detected using biotinylated secondary antibodies and avidin-biotin-peroxidase complex method was performed according to the manufacturer's instructions (DAKO LSAB 2 Kit). The reaction was stopped by washing with distilled water. Samples were counterstained with Mayer's hematoxylin for 30 sec and coverslipped with a permanent mounting medium. Control specimen exposed to PBS alone showed no specific staining.

Statistical analysis. The intensity of bands and blots was quantified measuring the optical density with an OneDscan computer software package. Data were analysed by unpaired Student's t-test. Differences were considered significant at $p<0.1$ (*) and $p<0.01$ (**).

Results

Elevated mRNA-expression for VEGF, Ang-1, Ang-2, VEGF-R1 and weak gene expression for Tie-2 and VEGF-R2. With the help of RT-PCR and the determination of the optical density we examined and quantified the gene expression of VEGF and its receptors VEGF-R1 and VEGF-R2, as well the receptor Tie-2 and its ligand Ang-1, Ang-2 in lesional skin of lipodermatosclerosis compared to normal skin (Fig. 1A-F). The significant increased mRNA expression in biopsies of lipodermatosclerosis is particularly clearly for VEGF ($p<0.01$), Ang-1 ($p<0.1$), Ang-2 ($p<0.1$) and VEGF-R1 ($p<0.01$). However, not statistical difference could be detected for Tie-2 and VEGF-R2 in lesional skin (LDS) in comparison with healthy skin (HS). In contrast, mRNA levels of the control gene ß-microglobulin were not altered in comparison with controls.

Elevated immunoreactivity for VEGF, Ang-1, Ang-2, VEGF-R1 and weak immunoreactivity for Tie-2 and VEGF-R2. The immunoreactive reaction and the determination of the optical density of blots were examined and quantified by immunoblotting for VEGF and its receptors VEGF-R1 and VEGF-R2, as well the receptor Tie-2 and its ligand Ang-1, Ang-2 in lesional skin of lipodermatosclerosis compared with normal skin (Fig. 2). For VEGF, Ang-1, Ang-2 and VEGF-R1 there were strongly enhanced protein expressions in lesional skin of lipodermatosclerosis (LDS) in comparison with healthy skin (HS). In contrast the protein expression of Tie-2 and VEGF-R2 did not display significant difference in biopsies of lipodermatosclerosis (LDS) in comparison with healthy skin (HS). Densitometric evaluation of the blot intensities between skin lesions of lipodermatosclerosis and healthy controls displayed significant differences for VEGF ($P<0.01$), Ang-1 ($p<0.1$), Ang-2 ($p<0.1$) and VEGF-R1 ($p<0.01$). No statistical differences were found for Tie-2 and VEGF-R2 between lesional skin and healthy skin.

Increased protein expression for VEGF, Ang-1, Ang-2 and VEGF-R1 in lesions of lipodermatosclerosis. The protein expression of VEGF, Ang-1 and Ang-2, Tie-2, VEGF-R1 and VEGF-R2 was determined in lesional skin of patients with lipodermatosclerosis and in normal human skin by immunohistochemistry (Figs. 3A-H).

Fig. 3A, C and D shows intense staining for VEGF, Ang-1, Ang-2, respectively, and weak staining for Tie-2 (Fig. 3E) in the deeper reticular dermis, particularly around tortous vessels in lipodermatosclerosis. Fig. 3F and H displays strong immunoreacitve staining for VEGF-R1 and weak staining for VEGF-R2 especially along the vessel structures in the reticular dermis in lesional skin of lipodermatosclerosis. Healthy skin in contrast displays weak protein expression for VEGF (Fig. 3B), Tie-2, VEGF-R1 (Fig. 3G) and VEGF-R2 in the epidermal and dermal layer of skin. Intensity of staining for VEGF, Ang-1, Ang-2, Tie-2, VEGF-R1 and VEGF-R2 in lesional skin was determined and is shown in Table II.

Discussion

Lipodermatosclerosis is highly associated with venous hypertension preceding venous ulceration of the lower extremities.

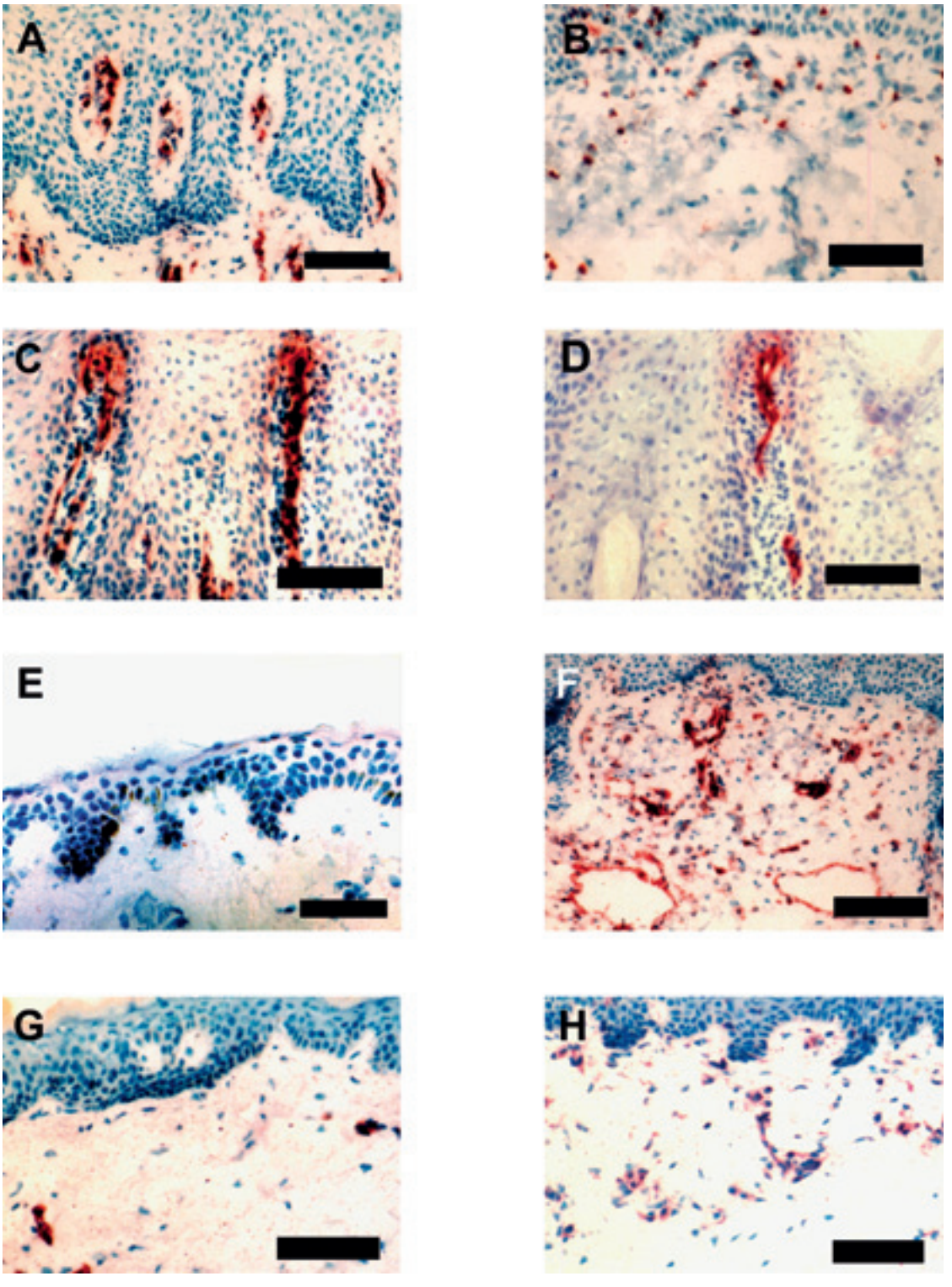

Figure 3. Immunohistochemical detection of VEGF, Tie-2, VEGF-R1 and VEGF-R2 in patients with lipodermatosclerosis in comparison with healthy skin. (A, C and D) intense staining is seen for VEGF, Ang-1 and Ang-2 in the deeper reticular dermis, particularly around fiber structures in tortous vessels in lipodermatosclerosis. Healthy skin in contrast displays weak protein expression for VEGF (B), Tie-2, VEGF-R1 (G) and VEGF-R2 in the epidermal and dermal layer of skin. The accumulation of cells in the deep reticular dermis and particularly along the newly developing vascular cells with strong signals for VEGF-R1 is shown in lipodermatosclerosis (F) in comparison with healthy skin (G). Weak immunohistochemical staining for VEGF-R2 in lipodermatosclerosis (H) and in healthy skin. Representative photographs from one patient is shown. The experiment was repeated with comparable results for lipodermosclerosis (n=4) and healthy skin (n=5). Scale bars are (A, B, C and D) 200 μm and (E, F, G and H) 100 μm.

Main histological features are avascularization and anti-proliferation of dermal microvascular endothelial cells with perivascular fibrin cuffs surrounding dermal capillary vessels, whereas venous ulcers are characterized by total loss of epidermal and partly dermal cellular layer (24). The underlying mechanisms leading to such drastic cellular changes of venous ulcerations have been a matter of debate. Pressure-damaged capillary vessels with leakage of fibrinogen or

Table II. Semi-quantitative evaluation of pro- and anti-angiogenic immunoreactivity in stained sections of venous leg ulcers.

Angiogenic agent	1	2	3	4	5
VEGF	++	+++	++	++	+++
Ang-1	++	+	++	++	++
Ang-2	+	+	+	+	+
Tie-2	-	+	-	-	+
VEGF-R1	+++	++	+++	++	++
VEGF-R2	-	-	-	-	+

Intensity of immunoreactivity was quantified as follows: no immunoreactive staining (-); low immunoreactive staining (+); median immunoreactive staining (++) and strong immunoreactive staining (+++). Five different specimens were stained with vascular VEGF, Ang-1, Ang-2, Tie-2, VEGF-R1 and VEGF-R2 and evaluated by three different observers. VEGF, vascular endothelial growth factor; Ang, angiopoietin; Tie-2, tyrosine kinase with immunoglobulin-like domains 2; VEGF-R, vascular endothelial growth factor receptor.

release of toxic metabolites by accumulated leukocytes or cytokine-mediated fibrin cuff formation have been suggested (25,26). Impairment of gas and nutritients exchange between blood and dermis has been supposed to be common features provoking ulcer formation.

Maintenance of the integrity of skin incorporates proliferating endothelial cells and supply of cellular components with nutritients and gas. Former studies showed, that VEGF is one of the most potent angiogenic agents. In spite of finding elevated levels of VEGF in the wound fluid in venous leg ulcers, there has been speculation as to how the angiogenic ligand receptor system is involved in the formation of venous leg ulcerations (21). Previous studies from our research team showed that degradation of the extracellular matrix plays a significant role and significantly influences the healing attitude of venous leg ulcers (27).

Directed proliferation, sprouting and migration of endothelia cells building up the supply chain for effective gas and nutritients supply requires interconnected signalling pathways. At the heart of this signalling network VEGF signals through ligand-binding to VEGF-R2 and Ang-1 through ligand-binding to Tie-2, hereby activating angiogenesis. In contrast decreased expression of VEGF-R1 has a disturbing effect on the balanced VEGF/VEGF-R2 signalling (28). In general, several studies suggest that Ang-1 is a proangiogenic factor that promotes angiogenesis and endothelial cell survival, especially in the presence of VEGF; whereas Ang-2 destabilizes vasculature that leads to apoptosis of endothelial cells and vessel regression in the presence or absence of VEGF (29).

Our experiments show for the first time an imbalanced angiogenic ligand receptor system for both VEGF and for angiopoietin. The mRNA-transcripts and blots for VEGF, Ang-1, Ang-2 and VEGF-R1 were significantly upregulated in all samples of lipodermatosclerosis in comparison with healthy skin by using reverse transcriptase-polymerase chain reaction and immunoblotting. Solely for Tie-2 and for VEGF-R2 no statistical difference could be detected in patients with lipodermatosclerosis in comparison with healthy skin. By immunohistochemistry we could confirm upregulated protein expression for VEGF, Ang-1, Ang-2 and VEGF-R1 compared with healthy skin. Staining of biopsies from lipodermatosclerosis displays strong immunoreactivity for VEGF in perivascular dermal vascular structures in comparison with normal skin. Interestingly, VEGF-R1 was seen at sites of disorganized cells, which would correspond to damaged cells within lesional tissue.

Therefore, elevated level of VEGF protein in biopsy specimen could be due to enhanced secretion from dermal cells in lipodermatosclerosis. Enhanced interaction of VEGF with VEGF-R2 usually promotes endothelial cell proliferation and vascularisation. Lack of VEGF binding to the VEGF-R2 receptor could further enhance inhibition of angiogenesis and hereby aggravate the supply of nutritients and gas in lipodermatosclerosis. However, in lipodermatosclerosis imbalanced ratio of VEGF-R1/VEGF-R2 could result in disturbed signalling pathway and the formation of tortous vessels, since VEGF-R1 acts as a negative regulator of VEGF-mediated angiogenesis and as a stimulator of pathological angiogenesis (11). Clinical signs of skin hardening in lipodermatosclerosis could be the result of enhanced avascularisation, which could be the consequence of an imbalanced VEGF and angiopoietin lingand receptor system.

Our data provide evidence that elevated Ang-2 expression on mRNA and protein level as well as an imbalanced Ang-1/Tie-2 system inhibited angiogenesis in lipodermato-sclerosis and hereby further accelerating the tissue break-down in lipodermatosclerosis. Ang-1 is known to support the integrity of endothelial cells, whereas Ang-2 has the opposite effect and promotes blood vessel destabilization as well as regression in the absence of the survival factor VEGF (14-16). It is conceivable that elevated levels of Ang-2 as an antagonist of angiogenesis could inhibit the proangiogenic action of Ang-1, which is elevated in lipodermatosclerosis. However, diminished expression of the angiopoietin receptor Tie-2 brings additional imbalance to the angiogenic system hereby disturbing the proliferation of endothelium in lipodermatosclerosis.

In summary, our data indicate lipodermatosclerosis to be characterized by an imbalanced ligand/receptor system for VEGF and angiopoietin. These data suggest that this imbalance on mRNA and protein level implies a disturbance in the dynamic balance of angiogenesis and its breakdown. Ulcer formation, therefore, may be favored by an enhanced

anti-angiogenic process in lipodermatoscle-rosis. Targeting this anti-angiogenic activity may provide a potential therapeutic strategy in the management of patients with advanced complications of chronic venous insufficiency.

Acknowledgments

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