Increased but imbalanced expression of VEGF and its receptors has no positive effect on angiogenesis in systemic sclerosis skin

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Abstract

Objective

To investigate the expression of vascular endothelial growth factor (VEGF) and its vascular and lymphatic receptors in skin in systemic sclerosis (SSc) compared to systemic lupus erythematosus (SLE), Raynaud's phenomenon (RP) and normal healthy control skin.

Methods

Staining was performed using rabbit anti-human antibodies in DAKO TechMate™ Horizon staining robot programmed for the biotin-streptavidin protocol.

Results

VEGF was sporadically and weakly expressed in normal skin, but in spite of vascular damage in diseased skin, VEGF expression was only slightly upregulated. In contrast, its vascular receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1), were clearly upregulated. Finally, the lymphatic VEGFR-3 (Flt-4) receptor was also upregulated in diseased skin and ectopically expressed also in blood vessels. Negative staining and positive sample controls confirmed the specificity of the staining.

Conclusion

The imbalanced expression of VEGF and its vascular receptors suggest that the compensatory efforts to angiogenesis fail in SSc, in part due to insufficient local production of VEGF, which was low compared to VEGFR expression. This is compatible with the recent observations on the lack of $\alpha V\beta 3$+ newly formed blood vessels in SSc skin. Since microvascular angiogenic stimuli normally induce first VEGF and then VEGFR, these findings also suggest that the angiogenic cascade is turned on, but there is a defect in the finalisation of its effects. Normalization of angiogenic cascade in SSc could provide a future therapeutic target.

Key words
Systemic sclerosis, angiogenesis, lymphangiogenesis.

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Introduction

Vascular involvement in systemic sclerosis (SSc) is well documented and has been known since the earliest descriptions of the disease. SSc is characterized by more or less specific microvascular changes such as "bushy" capillaries, hemorraghes and thrombosis gradually leading to diminished numbers of capillary blood vessels in affected sites, accompanied by fibrosis in the damaged area. The first target for vascular deterioration in SSc is endothelium (1, 2). Gradual vascular damages and blood vessel loss are among the most important components of the pathogenesis of SSc (1). The observation that SSc patient sera contain a factor cytotoxic for human endothelial cells was an important key to understanding the vascular changes (3, 4).

During the course of SSc also potent stimuli of angiogenesis are produced (5-8). VEGF and its three tyrosine kinase receptors (VEGFR1-3) are central molecules in the regulation of angiogenesis (9,10). Although VEGF is but one member of a family also comprising placenta growth factor PIGF, VEGF-B, VEGF-C and VEGF-D, it is the one, which is upregulated by hypoxia and/or hypoglycemia (9). VEGF stimulates angiogenesis and lack of it leads to the elimination of surplus blood vessels via vascular regression. Thus, VEGF adjusts the vascularity of tissue to its metabolic demands by an upregulation or downregulation of angiogenesis. However, the status of VEGF with respect to its receptors in SSc are not clear enough yet (1, 11).

The aim of this paper was to analyze expression of VEGF and its receptors in skin in patients suffering from SSc in comparison to systemic lupus erythematosus (SLE), Raynaud's phenomenon (RP) patients and healthy controls. We wanted to elucidate, whether the hypovascularity in advanced SSc is due to low/insufficient production of angiogenic stimulus (VEGF status) or lack of responsiveness of the endothelium (VEGFR status).

Patients and methods

Patients and biopsies

Forty patients (Table I) were examined clinically at Vilnius University Red Cross Hospital before a biopsy was taken. All patients were hospitalized and gave their informed consent. Patients with SSc (12) and SLE (13) met the classification criteria established by the American College of Rheumatology. All SSc patients had the diffuse form of systemic SSc. Six of the SSc patients had polyarthralgias, 4 esophageal dysmotility, 2 cutaneous ulcers, 2 heart involvement and sicca symptoms. None of the patients in this series had severe renal involvement or pulmonary hypertension. Capillaroscopy was not available. Patients with RP had had characteristic symptoms and no clinical or serological evidence of connective tissue disease (14). The healthy control skin samples were taken from patients in Surgical Department. Skin specimens were obtained from forearm skin. All biopsies were fixed in formalin and ethanol and embedded in paraffin.

Antibodies

The antibodies were purchased from Research Diagnostic Inc. (Flanders, NJ). The affinity purified IgG fractions of

Table I. Clinical characteristics of systemic sclerosis patients, comparators and controls studied.

Condition	No. of patients	Mean age[1]	Sex M/F	Duration of disease[1]
Scleroderma	9	47.5 ± 6.0 (29-54)	0/9	6.6 ± 5.4 (1-21)
SLE	10	40.3 ± 6.8 (25-63)	2/8	8.6 ± 5.7 (1-20)
RP	9	39.1 ± 7.4 (25-53)	0/9	5.9 ± 4.7 (1-20)
Control*	12	51.3 ± 13.0 (22-61)	1/11	-

[1]years,mean ± standard error of the mean, followed by range. SLE:systemic lupus erythematosus; RP: Raynaud's phenomenon; M: male; F: female. *Surgery Department patients, who did not suffer from any systemic diseases.

the antisera were used in the following concentrations: rabbit anti-human VEGF (RDI-VEGF N2abr) 2 µg/ml, rabbit anti-FLT-1 (human/rat/mouse) RDI-FLT1 CabR 2 µg/ml (recognizes VEGFR-1), rabbit anti-FLK-1 (human/rat/mouse) RDI-FLK1CabR 4 µg/ml (recognizes VEGFR-2),and rabbit anti-FLT-4 (human) RDI-FLT4CabR 0.5 µg/ml (recognizes VEGFR-3).

Immunohistochemistry

After extensive washing in 10 mM Tris-HCI buffred 0.9 M saline, pH 7.5 (TBS), sections were processed in DAKO TechMate™ Horizon instrument according to the instructions of DAKO ChemMate Detection Kit for the detection of VEGF and its receptors VEGFR-1 (Flt-1), VEGFR-2 (Flk-1), VEGFR-3 (Flt-4). Briefly, five µm thick paraffin sections were mounted on DAKO Capillary Microscope slides (TechMate™, Dako, Glostrup, Denmark), deparaffinized in xylene and rehydrated in a graded ethanol series and washed in 10 mM phosphate-buffered, 0.9 M saline, pH 7.4 (PBS). To reveal hidden epitopes, sections were pretreated with aqueous 0.4% pepsin in 0.1 normal HCI for 30 min at +37°C followed by extensive washing in TBS and preincubation in the primary antibodies for 90 min at +37°C in humidified chambers. After that the slides were washed with washing buffer and installed in DAKO TechMate™ Automated Immunostainer 500. All slides were automatically stained by the following protocol at +22°C: 1) the primary antibody, diluted with DAKO ChemMate™ antibody diluent, for 25 more minutes. 2) secondary antibody containing both biotinylated goat anti-rabbit IgG and biotinylated goat anti-mouse IgG antibodies for 30 minutes. 3) peroxidase block for 25 minutes. 4) peroxidase-conjugated streptavidine 3 times for 3 minutes. 5) HRP Substrate Buffer and finally 6) substrate working solution containing 3,3'-diaminobenzidine tetrahydrochloride (ChemMate™ detection kit) for 5 minutes. Between each step, the sections were washed with DAKO ChemMate™ washing buffers 3 times and dried in absorbent pads. Finally, all sections were dehydrated in an increasing ethanol series, cleared in xylene, and mounted in synthetic mounting medium (Diatex, Beckers Industrifärg, Märsta, Sweden).

Normal rabbit IgG was used at the same concentrations as and instead of the primary antibodies as negative staining controls. Consecutive sections were counterstained with hematoxylin or left without counterstaining.

Microscopic assessment and documentation

Microscopic assessment was done using a low light-charge screen mounted with a 12-bit PC digital image camera (SensiCam, Kelheim, Germany) on a Leitz Diaplan light microscope (Wetzler, Germany). The whole section areas were analyzed. Two independent observers did the analysis, after a joint training session, with practically same results, without knowledge of the patient's diagnosis or clinical data. The number of positively staining vascular profiles was graded subjectively to 0 = no profiles; ± = very few profiles; + = few profiles; ++ = moderate numbers of profiles; and +++ = high numbers of profiles. A similar scale was used for VEGF, VEGFR-1, VEGFR-2 and VEGFR-3. This scoring system did not take the intensity of staining into consideration, but some comments are made also on the intensity of staining in the Results section. Photos were made by digital processing and color printer.

Results

VEGF

Our data of immunohistochemical staining showed that there were only very few or generally a total lack of VEGF immunoreactive profiles in adult control skin. The number of VEGF immunoreactive profiles was a slightly higher in SSc and SLE than in Raynaud's phenomenon and in healthy control subjects, in this ranking order (Table II). When expressed, VEGF was present in the epidermis and in its derivatives, and in media and adventitia of some dermal blood vessels (Fig. 1a). Blood platelets and some stromal cells were occasionally VEGF positive.

Vascular VEGF receptors (VEGFR-1 and VEGFR-2)

Structures displaying VEGF receptor staining were usually much more common than those displaying staining for the VEGF itself (Table II). Microvasculature of the skin in SSc, SLE and RP patients displayed moderate numbers of VEGFR-1 positive vascular profiles (Fig. 1b), whereas blood vessels in subjects with normal skin only had a few positively stained profiles, although some patients at some sites displayed moderate numbers of VEGFR-1 positive vascular profiles. There were few or even moderate numbers of VEGFR-2 immunoreactive profiles in skin microvasculature in SLE, RP and SSc, but very few or at most few such profiles in the normal control skin (Table

Table II. Expression of vascular endothelial growth factor VEGF and its receptors in skin biopsies in systemic sclerosis, systemic lupus erythematosus (SLE), Raynaud's phenomenon and healthy controls.

Condition	VEGF	VEGFR-1 (=FLT-1)	VEGFR-2 (=FLK-1)	VEGFR-3 (=FLT-4)
Scleroderma (n = 9)	±/+	++	+	++ / +++
SLE (n = 10)	± /+	++	++	++
Raynaud's phenomenon (n = 9)	0 / +	++	+ / ++	+++
Healthy controls* (n=12)	0 / ±	+ / ++	± / +	+ / ++

Score value: 0 = no immunostained profiles; ± = very few profiles; + =few profiles; ++ = moderate number of profiles; +++ = high number of profiles. *Surgery Department patients not suffering from systemic diseases.

II). Also VEGFR-2, like VEGFR-1, was in dermis localized to microvascular endothelium (Fig. 1c).

Lymphatic VEGF receptors (VEGFR-3)

VEGFR-3 immunoreactive profiles were seen in moderate or high numbers in dermis in RP, SSc and SLE, in this ranking order. Control skin samples contained few or moderate numbers of VEGFR-3 immunoreactive profiles (Table II). Surprisingly, some vascular profiles were labeled in VEGFR-3 staining (Fig. 1d). This was confirmed by demonstration of VEGFR-3 immunoreactive profiles, which in consecutive sections were shown to be PAL-E reactive blood vessels rather than lymphatic vessels (data now shown; see also Paavonen *et al.*, in press).

Staining controls and other observations

At the IgG concentrations used for staining, negative staining controls were totally negative and, thus, confirmed the specificity of the staining. Even in patients and at sites, where vascular and/or lymphatic profiles lacked VEGF staining, the skin and its appendices stained positively for VEGF, which served as an internal, positive staining control. Usually VEGF and its receptors were positively/more strongly stained at sites with local inflammatory cell infiltrates. Staining of consecutive sections showed that many VEGFR immunoreactive vascular profiles were VEGF negative. We did not observe any apparent differences in VEGF or VEGF receptor expression as a function of disease duration in SSc or any of the other rheumatic diseases analyzed except for the hypovascularity, loss of dermal papillae and dermal fibrosis seen in advanced SSc skin. Similarly, there were no apparent correlations between the extent of teleangiectasiae or other clinical characteristics and VEGR or its receptor expression.

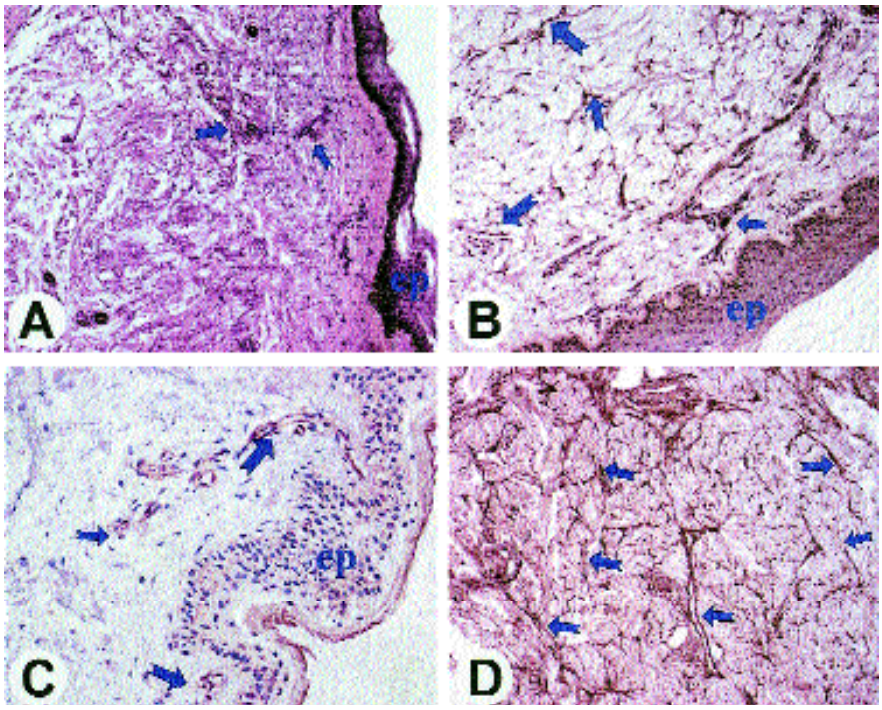

Fig. 1. Immunohistochemical localization of VEGF and its receptors in skin in systemic sclerosis.
A. Very few and faintly staining VEGF; immunoreactive blood vessel profiles (arrows) in skin in systemic sclerosis. Some cells of epidermis and stroma are also immunoreactive. ep = epidermis. Counterstained with hematoxylin. The original magnification x250.
B. Immunolocalization of VEGFR-1 (Flt-1) in skin in systemic sclerosis. Moderate numbers of positively staining vascular profiles can be seen (arrows) in the papillary and reticular layers of the dermis. Counterstained with hematoxylin. The original magnification x250.
C. Immunolocalization of VEGFR-2 (Flk-1) in skin in systemic sclerosis. Some VEGFR-2 immunoreactive vascular profiles have been marked with arrows. Counterstained with hematoxylin. The original magnification x250.
D. Immunolocalization of VEGFR-3 (Flt-4) in skin in systemic sclerosis. High numbers of VEGFR-3 immunoreactive lymphatic and blood vessels (arrows) can be seen in dermis in systemic sclerosis. The original magnification x250.

Discussion

Angiogenesis refers to sprouting or splitting of capillaries from pre-existing vessels. In adults, angiogenesis is usually limited to female reproductive system, wound healing and tumourigenesis (9,10). The stimuli normally driving quiescent microvasculature to an activated, angiogenic state are hypoxia and hypoglycemia. Because VEGF is induced by these two stimuli, it has been suggested to represent the coupling factor between the local tissue needs/supply of oxygen and glucose on one hand and the angiogenesis or vascular regression (pruning) on the other hand. Indeed, it seems that VEGF is perhaps the most potent multifunctional cytokine reprograming endothelial cell gene expression towards angiogenesis (9, 10), whereas lack of it induces apoptosis of the endothelial cells and regression of surplus blood vessels. Due to vascular damage (1,15-17), hypovascularity (18,19) and low transcutaneous oxygen tension (20, 21), one would expect strong and extensive induction of VEGF in SSc skin (22, 23) and, thus, many VEGF immunoreactive profiles. However, our findings demonstrate that induction of VEGF is relatively weak so that the VEGF immunoreactive vascular profiles are few in SSc skin. In comparison to VEGFR immunoreactive responder cells the VEGF producer cells were very few, which would prevent effective paracrine communication between VEGR producing cells and its target endothelial cells. Finally, the hypovascularity developing in advanced SSc clearly demonstrates that the compensatory mechanisms responsible for the maintenance and/or sprouting of new vessels fail in SSc. Insufficient induction of VEGF might be responsible for this failure to maintain the skin microvasculature in SSc.

Defective angiogenesis and/or mainte-

nance of already formed microvessels might not exlusively be due to lack of VEGF, but mainly to unresponsiveness of the endothelial cells to its programming effects. Because VEGF effects on blood vessels are mediated via two tyrosine kinase receptors, namely VEGFR-1/Flt-1 and VEGFR-2/Flk-1 (10), it was of interest to assess the VEGFR phenotype of microvascular endothelial cells in SSc skin. In contrast to VEGF immunoreactive vascular profiles, which were only few or very few, these two VEGFR immunoreactive vascular profiles were present in relatively high numbers. This was true when VEGFR immunoreactivity was compared to healthy control skin (i.e. induction compared to normal skin) or to VEGF expression (i.e. the VEGFR ligand). These findings suggest that the lack of maintenance and/or angiogenesis in SSc is not caused by lack of target cell responsiveness: their VEGFR positive phenotype indicates that they would be susceptible to VEGF effects would VEGF be present. Interestingly, usually VEGF and VEGFR are both upregulated by hypoxia and, furthermore, VEGF is induced before VEGFR. Therefore, the very presence of VEGFR expression suggests that the angiogenic cascade is turned on, but that there is a defect in VEGF epxression rather than in the VEGFR expression. Interestingly, newly formed "immature" blood vessels, in contrast to mature blood vessels with well-developed periendothelial cells, are particularly sensitive to VEGF deprivation (24). In accordance with these findings, we have recently shown that such newly formed, $\alpha V\beta 3$ integrin receptor positive blood vessels are almost totally lacking in SSc skin (Konttinen *et al.*, submitted). As to VEGF/VEGFR status, SSc was relatively similar to SLE and RP, which suggests that there are some SSc specific but as yet undefined factors, which contribute to loss of microvasculature in SSc skin in the long term. The observation that SSc patient sera contain a factor cytotoxic for human endothelial cells may be an important clue (4).

In contrast to VEGFR-1 and VEGFR-2, VEGFR-3 is largely restricted to lymphatic endothelium (25). The state of the lymphatic vessels affects interstitial edema, accumulation of pro-inflammatory/pro-fibrotic stimuli and lymphocyte recirculation. We found VEGFR-3 expression in some lymphatic vessels, but also in vascular profiles. This finding is compatible with our recent findings, which suggest that also in inflammatory synovitis blood vessels may undergo transformation to an embryonic VEGFR-3 positive phenotype leading to an ectopic VEGFR-3 expression also in blood vessels as recognized with staining using PAL-E and laminin antibodies (26).

The expression of VEGF and its receptors in SSc skin and plasma needs further attention (23). Confounding factors include medication, platelet-derived VEGF and other angiogenic stimuli. Nonsteroid anti-inflammatory drugs (NSAID) inhibit angiogenesis through direct effects on endothelial cells, and inhibition of both COX-1 and COX-2 play a pivotal role in this process (27). Alpha-granules of blood platelets contain VEGF and anti-inflammatory drugs interfere with the process of VEGF discharge (28). The effect of local oxygen tension on endothelial and periendothelial cells need to be assessed.

Taken together, our observations on VEGF and VEGFR-1, -2 and -3 expression in skin suggest that the loss of microvasculature in SSc is explained in part by lack of VEGF, not its receptors. It would seem that this would make SSc an amenable target for VEGF therapy, because 1) VEGF production is defective (with respect to VEGFR expression), 2) the target cells express VEGFR and 3) the vascular endothelium is easily accessible via the blood circulation. Such therapies are already being developed for cancer treatment, tissue engineering and other purposes (29-32).

References

1. HERRON GS, ROMERO LI: Vascular abnormalities in scleroderma. *Semin Cutan Med Surg* 1998; 17: 12-7.
2. MACKIEWICZ Z, TAMULAITIENE M, KIRDAITE G, WOOLLEY D, JEZIORSKA M,DÁBROS W: Early vascular damages in systemic sclerosis. *Acta Med Lituanica* 1999; 6: 3-6.
3. SPROTT H, MULLER-LADNER U, DISTLER O *et al.*: Detection of activated complement complex C5b-9 and complement receptor C5a in skin biopsies of patients with systemic sclerosis (scleroderma). *J Rheumatol* 2000; 27: 402-4.
4. COHEN S, JOHNSON AR,HURD E: Cytotoxicity of sera from patients with scleroderma. Effects on human endothelial cells and fibroblasts in culture. *Arthritis Rheum* 1983; 26: 170-8.
5. GRASSI W, MEDICO PD, IZZO F, CERVINI C: Microvascular involvement in systemic sclerosis: capillaroscopic findings. *Semin Arthri - tis Rheum* 2001; 30: 397-402.
6. RIBATTI D, CANTATORE FB, VACCA A, *et al.*: Systemic sclerosis stimulates angiogenesis in the chick embryo chorioallantoic membrane. *Clin Rheumatol* 1998; 17: 115-20.
7. MAJEWSKI S, SKOPINSKA-ROZEWSKA E, JABLONSKA S *et al.*: Modulatory effects of sera from scleroderma patients on lymphocyte-induced angiogenesis. *Arthritis Rheum* 1985; 28: 1133-9.
8. FLEISCHMAJER R, PERLISH JS: ^{3}H-thymidine labeling of dermal endothelial cells in scleroderma. *J Invest Dermatol* 1977; 69: 379-82.
9. DOR Y, PORAT R, KESHET E: Vascular endothelial growth factor and vascular adjustments to perturbations in oxygen homeostasis. *Am J Physiol* 2001; 280: C1367-74.
10. OLOFSSON B, JELTSCH M, ERIKSSON U, ALITALO K: Current biology of VEGF-B and VEGF-C. *Curr Opin Biotechnology* 1999; 10: 528-35.
11. KUROKI M, VOEST E, AMANO S, *et al.*: Reactive oxygen intermediates increase VEGF expression *in vitro* and *in vivo*. *J Clin Invest* 1996; 98: 1667-75.
12. MASI AT, RODNAN GP, MEDSGER TA JR *et al.*: Preliminary criteria for the classification of systemic sclerosis (scleroderma). Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. *Arthritis Rheum* 1980; 23:581-90.
13. TAN EM, COHEN AS, FRIES JF *et al.*: The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; 25: 1271-7.
14. LEROY EC,MEDSGER TA JR: Raynaud's phenomenon:A proposal for classification. *Clin Exp Rheumatol* 1992; 10: 485-8.
15. PIGNONE A, SCALETTI C, MATUCCI-CERINIC M *et al.*: Anti-endothelial cell antibodies in systemic sclerosis:Significant association with vascular involvement and alveolo-capillary impairment. *Clin Exp Rheumatol* 1998; 16: 527-32.
16. BLANN AD, ILLINGWORTH K, JAYSON MI: Mechanisms of endothelial damage in systemic sclerosis and Raynaud's phenomenon. *J Rheumatol* 1993; 20: 1325-30.
17. KAHALEH MB, SHERER GK, LEROY EC: Endothelial injury in scleroderma. *J Exp Med* 1979; 149: 132-5.
18. BOLLINGER A, JAGER K, SIEGENTHALER W: Microangiopathy of progressive systemic sclerosis: Evaluation by dynamic fluorescence videomicroscopy. *Arch Intern Med* 1986; 146: 1541-5.
19. MARICQ H, LEROY E, D'ANGELO W: Diag-

nostic potential of *in vivo* capillary microscopy in scleroderma and related disorders. *Arthritis Rheum* 1980; 23: 183-9.
20. VALENTINI G, LEONARDO G, MOLES DA *et al.*: Transcutaneous oxygen pressure in systemic sclerosis: evaluation at different sensor temperatures and relationship to skin perfusion. *Arch Dermatol* 1991; 283: 285-8.
21. SILVERSTEIN JL, STEEN VD, MEDSGER TA JR, FALANGA V: Cutaneous hypoxia in patients with systemic sclerosis (scleroderma). *Arch Dermatol* 1988; 124: 1379-82.
22. DISTLER O, DEL ROSSO A, SCHEID D *et al.*: Overexpression of vascular endothelial growth factor (VEGF) and its receptors in systemic sclerosis (SSc): Evidence for protective effects of VEGF against the development of finger ulcers. *Ann Rheum Dis* 2001; 60: S78.
23. KIKUCHI K, KUBO M, KADONO T, YAZAWA N, IHN H, TAMAKI K: Serum concentration of vascular endothelial growth factor in collagen diseases. *Br J Dermatol* 1998; 139: 1049-51.
24. BENJAMIN LE, HEMO I, KESHET E: A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. *Development* 1998; 125: 1591-8.
25. LARRIVEE B, KARSAN A: Signaling pathways induced by vascular endothelial growth factor. *Int J Mol Med* 2000; 5: 447-56.
26. PAAVONEN K, MANDELIN J, JUSSILA L *et al.*: Vascular endothelial growth factors -C and -D and their VEGFR-2 and -3 receptors in healthy and arthritic synovium. *J Rheumatol* (in press).
27. JONES MK, WANG H, PESKAR BM *et al.*: Inhibition of angiogenesis by nonsteroidal antiinlflammatory drugs:insight into mechanisms for cancer growth and ulcer healing. *Nat Med* 1999; 5: 1418-23.
28. WARTIOVAARA U, SALVEN P, MIKKOLA H *et al.*: Peripheral blood platelets express VEGF-C and VEGF which are released during platelet activation. *Thromb Haemost* 1998; 80: 171-5.
29. TAKESHITA S, ZHENG LP, BROGI E *et al.*: Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. *J Clin Invest* 1994; 93: 662-70.
30. PIGNONE A, GENERINI S, MATUCCI CERINIC M: Prostaglandin E_1 restores the level of vWF and ACE in chronic critical limb ischemia in systemic sclerosis. *Clin Exp Rheumatol* 2001; 19: 358-9.
31. FURST DE: Rational therapy in treatment of systemic sclerosis. *Curr Opin Rheumatol* 2000; 12: 540-4.
32. FURST DE,CLEMENTS PJ: Hypothesis for the pathogenesis of systemic sclerosis. *J Rheumatol* 1997; 48 (Suppl.): 53-7.