Evaluation of serum vascular endothelial growth factor and endostatin in systemic sclerosis patients – correlation with lung and cardio-vascular system involvement

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Abstract

Recent studies point out the role of angiogenesis disturbances in the development of systemic sclerosis (SSc).

The aim of our study was to examine vascular endothelial growth factor (VEGF) and endostatin serum levels in scleroderma patients and their correlation with skin involvement and internal organ changes.

We studied 33 patients (14 with limited and 19 with diffuse SSc). The extension of skin involvement was measured using Total Skin Score (TSS). Internal organ involvement was assessed with specialistic procedures. ELISA measured serum VEGF and endostatin concentrations.

We found statistically significant difference between endostatin serum levels in the control group and the patients with SSc and dSSc. Statistical analysis showed that higher VEGF levels were found in SSc and lSSc patients with changes on chest X-ray examinations, higher endostatin levels were more often observed in SSc and lSSc patients with abnormalities in cardiovascular system and in lSSc with lung dysfunction.

The obtained results revealed that disturbances of angiogenesis might play a role in SSc pathogenesis. VEGF serum levels correlate with changes on chest X-ray and endostatin serum levels with abnormalities in cardiovascular system and lung dysfunction.

Key words: *VEGF, endostatin, SSc, lung and cardiovascular system involvement*

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Introduction

Scleroderma (Sclerodermia Systemica, Systemic Sclerosis, SSc) is a multisystem connective tissue disease with small and larger vessel involvement, which may trigger autoimmunological processes and antinuclear antibodies (ANA) production. Scleroderma is characterized by excessive collagen and other extracellular matrix components production both in the skin and internal organs [1]. Those processes can lead to severe disability or even death [2].

Literature data point out at the importance of angiogenesis regulatory factors in pathogenesis of systemic sclerosis [3, 4]. Angiogenesis i. e. development of new blood vessels is a crucial process in both physiology including embryogenesis, menstrual cycle, decidua formation, hair growth cycle, wound healing and pathology including neoplasms and metastases development, rheumatoid arthritis, diabetic retinopathy, psoriasis vulgaris, endometriosis and connective tissue diseases [5, 6].

Experimental data demonstrated that angiogenesis could be influenced by serum of SSc patients. This process depends on the stage and type of the disease and the balance between pro- and antiangiogenic factors [7].

The following substances are considered to stimulate angiogenesis: basic fibroblast growth factor (bFGF) [8, 9], interleukin 18 (IL-18) [10] and vascular endothelial growth

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factor (VEGF). VEGF is an angiogenic and mitogenic glicoprotein which increases blood vessel permeability via two selective endothelial cells surface receptors [6]. VEGF after receptor binding, stimulate endothelial cell proliferation, plasminogen activation, induces α and β integrin subunits expression and leads to changes in collagenase activation [6]. Literature data showed that this cytokine could influence proliferation, survival as well as migration of endothelial cells [11, 12]. Changes in endothelial cells in the course of systemic sclerosis, are one of key factors in the development of progressive fibrosis of the skin and internal organisms. It was demonstrated that destruction of some endothelial cells can further stimulate destruction of the other cells. Subsequently, production of different cytokines and adhesion molecules, which activate fibroblasts situated in the vicinity of blood vessels is observed [13].

VEGF plays an important role in many diseases characterized by tissue hypoxia where its expression in greatly enhanced [14-16]. It was demonstrated that angiogenesis observed in neoplastic processes many be stimulated by VEGF and thus responsible for disease progression [17, 18]. Favorable effect of VEGF was found only in a very few cases of peripheral blood vessel diseases and ischaemic heart disease [19].

It was demonstrated that VEGF serum levels correlated with disease activity in both rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [20].

However, knowledge on the role of VEGF in SSc is considered to be rather inconsistent. On the one hand, VEGF can exert a favorable effect by prevention of digital phalanges ulcer development, but on the other hand it can be responsible for disease severity and progression [21, 22].

Blood vessel atrophy is often observed on capilaroscopy in SSc patients presenting ulcerations. It was observed that inhibition of angiogenesis may be triggered by monocytes and lymphocytes obtained from SSc patients [23, 24]. The following substances exert an inhibitory effect on angiogenesis: angiostatin, endostatin, some interleukins (IL-1, IL-4, IL-10, IL-12), interferons, tissue inhibitors of metalloproteinase 1, 2 and 3 (TIMP-1, -2, -3) as well as angiopoietin 2 and thrombospondin 1 and 2 [25].

Endostatin is a non-collagenic domain of collagen XVIII, of 20 kDa mollecular weight [26]. Considerable amounts of this protein are found in the blood vessels of the lungs and the skin, which are the organs most frequently involved in SSc patients. Collagen XVIII is produced by fibroblasts and hepatocytes and its proteolytic fragment – endostatin is easily found in the circulation after being processed by catepsin L from perivascular collagen [27]. This protein can be found in the blood, urine and basement membranes of many internal organs [28]. Role of endostatin has not been fully elucidated. However, it was demonstrated that endostatin exert a selective inhibitory effect on endothelial cell proliferation stimulated by bFGF [29]. It also decreases activation of proenzymatic metalloproteinase 2 and catalytic properties of membrane metaloproteinases type 1 and 2 [30]. Endostatin can considerably reduce endothelial cell migration to newly developed basement membrane stimulated by VEGF [30]. Endostatin was quite well investigated in neoplastic diseases [25]. Some literature data point at favourable effect of endostatin administration, as a recombinant protein, to the patients with solid tumors [31]. It was observed that incubation of endothelial cells with endostatin leads to cell apoptosis [32, 33].

Studies on the role of endostatin in connective tissue diseases did not find and correlation between this protein serum levels and activity of the disease. Increased endostatin levels were observed in SSc patients in comparison to the control group [4]. Disturbances in VEGF and endostatin production were also observed in patients with rheumatoid arthritis [34].

The above-described data on the role of both VEGF and endostatin, which inevitably exert some role on angiogenesis in scleroderma, prompted us to evaluate both proteins levels in serum of 33 systemic sclerosis patients. Detailed statistical analysis was performed to describe any correlations between protein levels and internal organ changes in the examined group of patients.

Material and methods

Our study involved 33 SSc patients, 28 women and 5 men, age range 16-69 years. In our group 19 patients presented limited SSc – lSSc (mean age 52.7±12.8 years) and 15 diffuse form – dSSc (mean 45.5±12.6 years). All the patients were diagnosed according to ARA criteria [35]. Because of the rapid disease progression, all the dSSc patients were on immunosupressive regimen (50 mg cyclophosphamide daily and 20 mg prednison daily). The patients with lSSc were never put on these drugs. All patients also received channel blockers or pentoxifilline and vitamin E in comparable doses.

Duration of Raynaud's phenomenon and skin sclerosis were evaluated in all the patients. The degree of sclerosis of the skin was assessed using the Total Skin Score (TSS) according to Kahaleh et al. (0-66 points) [36].

Basic laboratory tests, including erythrocyte sedimentation rate (ESR), full blood count, urine tests, urea and creatinine levels were performed in all the patients. The following additional examinations were also performed in our group of patients: oesophagal scyntigraphy, 24-hour ECG monitoring, Doppler echocardiography, chest X-ray, X-ray of feet and hand bone, lung spirometry. The letter was evaluated in percentages of the required value: Forced Vital Capacity (FVC), Forced Expiratoy Volumen in 1 sec (FEV_1) and the ratio FEV_1/FVC. Restrictive disturbances of pulmonary changes were diagnosed when FVC was below 80% of the desired value, while the value of the ratio FEV_1/FVC was elevated or within the normal range. Obstructing lesions were diagnosed when the value of FEV_1 was reduced, and mixed lesions when the reduction in the predicted values of both FVC and FEV_1 were similar [37].

Table 1. Characteristics of the examined patients with systemic sclerosis

	Systemic sclerosis (SSc)	Limited systemic sclerosis (lSSc)	Diffuse systemic sclerosis (dSSc)
Raynaud's phenomenon duration (years)	11.52±9.67	15.84±10.98*	6.06±2.84
Disease duration (years)	4.44±3.83	5.47±4.38*	3.13±2.58
Total Skin Score (TSS) (points)	28.14±13.75	17.78±5.13*	41.26±9.04

** differences statistically significant at p<0.05 – comparison limited versus diffuse systemic sclerosis*

Antinuclear antibodies serum levels (ANA) were identified by the indirect immunofluorescence method (IIF), using HEp-2 cells as a substrate (Sigma Diagnostics). The precise identification of antibodies was performed by the double immunodiffusion method (DID) in agar gelatin according to Outchterlony [38].

Blood was collected in the morning into the pyrogen--free tubes and stored in -20ºC until further evaluated. Serum levels of VEGF and endostatin were evaluated by immunoenzymatic method (ELISA). Commercially available kits were employed (Quantikine R&D Systems Inc, USA). Concentrations were calculated using a standard curve generated with specific standards provided by the manufacturer. The minimum detecteble concentration of VEGF was less than 9.0 pg/ml and endostatin 1.953 ng/ml.

VEGF levels were evaluated in 33 patients (14 with dSSc and 19 with lSSc) and endostatin in 30 patients (14 with dSSc and 16 with lSSc).

The control group consisted of 20 healthy persons (15 women, 5 man; aged 25-64 years, mean age 46.3±13.2 years).

All the patients and individuals from the control group gave their informed consent to participate in our study according to the requirements of the Bioethic Committee of Medical University of Lodz.

Statistical analysis

The obtained results were expressed as mean, maximum, minimum, and median values together with standard deviation. Numerical variables distribution was assessed by Shapiro-Wilk test, Mann-Whitney, Cochran-Cox and two independent sample tests were employed for comparison of mean values. Correlation between numerical values was evaluated by Spearman rang correlation coefficient – ρ. A p value less than 0.05 was considered to be statistically significant.

Results

Characteristics of the patients are presented in Table 1. Table 2 demonstrated abnormalities and changes in internal organs found in the examined group of patients.

The mean value of VEGF in systemic sclerosis patients was 194.0±196.8 pg/ml and in control group 271.2±201.0 pg/ml, in lSSc patients was 219.5±228.4 pg/ml and in dSSc patients 159.6±144.6 pg/ml (Table 3). The differences were not statistically significant (p>0.05).

The mean value of endostatin in systemic sclerosis patients was 113.6±60.7 ng/ml and in the control group 73.6±25.9 ng/ml (Table 3). The difference was statistically significant (p=0.003). Statistically significant difference was also found between endostatin serum levels in the control group and the patients with and dSSc group (p=0.007). The differences in the mean value in both subgroups were not statistically significant (Table 3).

We have not found any statistically significant correlations between VEGF and endostatin serum levels and Raynaud's phenomenon duration, disease duration, skin lesion severity and number of internal organ involved (Table 4).

Statistical analysis showed that higher VEGF levels were found in patients with changes on chest X-ray examinations (systemic sclerosis – p=0.02; limited SSc – p=0.002), whereas higher endostatin levels were more often observed in patients with disturbances in cardiovascular system (systemic sclerosis – p=0.01; lSSc p=0.006) and disturbed lung function (lSSc – p=0.03) (Table 5).

Discussion

The process of angiogenesis has been much wider studied in neoplastic processes than in connective tissue diseases [39]. However, there is some data on angiogenesis disturbances in the course of autoimmunological processes [40, 41].

Ordinary tissue hypoxia can lead to new blood vessel formation [19]. In scleroderma, on histopathology one can observe perivascular inflammatory infiltrate, diminishing the number of blood vessels and narrowing of their lumen together with excessive deposition of extracellular matrix components [21]. However, despite decreased blood flow and reduced partial oxygen pressure in scleroderma, there is no evidence of proper new blood vessel formation in the skin [22].

It was suggested that VEGF levels may reflect disease activity and that there is some correlation between its serum concentration and lung fibrosis [42]. Viac et al., however, did not observe such correlation [43]. The majority of research groups report that serum VEGF levels in SSc patients are increased in comparison to the control group [3]. In the examined group of patients mean VEGF levels were demonstrated to be lower when comparing to the control group. However, some patients presented higher levels than the control group. Lower VEGF levels were observed in

Table 2. Abnormalities in laboratory tests and internal organs observed in the examined group of patients

		Systemic Sclerosis n=33	limited Systemic Sclerosis n=19	diffuse Systemic Sclerosis n=14
Oesophagus scitigraphy	Normal	7	4	3
	Disturbed	26	15	11
Heart (ECG and Doppler echocardiography)	Normal	19	12	7
	Abnormal	13	7	7
X-ray hand and feet bone	Normal	8	4	4
	Abnormal	25	15	10
X-ray chest	Normal	21	14	7
	Abnormal	12	5	7
Spirometry	Normal	13	9	4
	Disturbed	20	10	10

– oesophagal scyntigraphy disturbed: slowed down passage in the lower and/or central part of the oesophagus
– cardio-vascular abnormalities: tendency to tachyarrhythmias, lower potentials of different parameters describing variability of cardiac rythmus or conduction system abnormalities, ventricular arrhythmia, silent ischaemic episodes, diastolic left ventricular disturbances, valvular lesions
– X-ray feet and hand bone abnormalities: juxta-articular demineralisation, joint space narrowing in digitals
– X-ray chest abnormalities: symmetrical fibrosis of the lung base and „honey-comb" picture
– Spirometry disturbed: lung function disturbances of restrictive, obstructive or mixed type

Table 3. Serum levels of vascular endothelial growth factor (VEGF) (pg/ml) and endostatin (ng/ml) in systemic sclerosis patients and the control group

	vascular endothelial growth factor				Endostatin			
	Systemic Sclerosis n=33	limited Systemic Sclerosis n=19	diffuse Systemic Sclerosis n=14	control group n=10	Systemic Sclerosis n=30	limited Systemic Sclerosis n=16	diffuse Systemic Sclerosis n=14	control group n=20
Mean	194.0	219.5	159.6	271.2	113.6*	101.9	127.1**	73.6
±SD	196.8	228.4	144.6	201.0	60.7	53.1	67.8	25.9
Me	138.2	138.2	134.3	286.35	107.2	101.4	124.2	77.8
Range	0-836.4	0-836.4	0-455.6	23.7-708.5	13.6-261.2	13.6-209.6	18.0-261.2	18.0-110.4

** comparison with the control group – statistically significant at p=0.003*
*** comparison with the control group – statistically significant at p=0.007*

dSSs patients than lSSc ones, which is not in agreement with Distler et al. [3]. Those authors suggested some correlation between higher VEGF levels and disease activity together with disturbed angiogenesis, which may be a proof of biological blockage development of pro-angiogenic activity of VEGF in the systemic sclerosis patients. The role of VEGF receptors in the course of angiogenesis has not been elucidated in our patients. It is widely accepted that many different factors such as hypoxia, interleukin 1, transforming growth factor beta and platelet-derived growth factor influence VEGF activity. In lSSc group we observed that the lower serum VEGF level, the more severe skin fibrosis, whereas in dSSc group the higher VEGF level the more severe skin fibrosis. It is difficult to explain the above differences. Maybe they result from different VEGF levels observed in patients with different duration of Raynaud's phenomenon and duration of the disease itself. In dSSc group the shorter Raynaud's phenomenon and disease duration the higher VEGF levels were found in contrast to the lSSc group. Our results seem to support the hypothesis of dual function of VEGF in pathogenesis of systemic sclerosis. On the one hand, VEGF can exert a favorable effect on blood vessels on the other hand, it can precipitate skin fibrosis development [3]. In RA patients median serum levels of VEGF were comparable with those found for SSc patients in Distler et al study [3]. Some authors observed that bloc-

Table 4. Correlations between serum vascular endothelial growth factor (VEGF) (pg/ml) and endostatin (ng/ml) levels in SSc patients and duration of Raynaud's phenomenon, skin involvement and extensiveness of skin involvement and number of internal organs involvement

	vacular enothelial growth factor			**Endostatin**		
	Systemic Sclerosis n=33	**limited Systemic Sclerosis n=19**	**diffuse Systemic Sclerosis n=14**	**Systemic Sclerosis n=30**	**limited Systemic Sclerosis n=16**	**diffuse Systemic Sclerosis n=14**
			Duration of Raynaud's phenomenon			
ρ	0.231	0.389	-0.087	-0.143	0.156	-0.164
p	**>0.05**	**>0.05**	**>0.05**	**>0.05**	**>0.05**	**>0.05**
			Duration of skin involvement			
ρ	0.149	0.294	-0.098	0.026	0.339	-0.123
p	**>0.05**	**>0.05**	**>0.05**	**>0.05**	**>0.05**	**>0.05**
			Total Skin Score			
ρ	-0.193	-0.158	-0.297	0.311	0.239	0.091
p	**>0.05**	**>0.05**	**>0.05**	**>0.05**	**>0.05**	**>0.05**
			Number of internal organs involved			
ρ	0.238	0.354	0.215	0.200	0.341	-0.080
p	**>0.05**	**>0.05**	**>0.05**	**>0.05**	**>0.05**	**>0.05**

kade of VEGF reduced the disease severity in murine collagen-induced arthritis [44, 45].

Statistically significant difference between VEGF levels and internal organ changes was found in all the examined lSSc subgroup. Higher VEGF levels correlated with changes observed on chest X-ray which is in agreement with the results of Kikuchi et al. [42]. This group suggested that VEGF, via endothelial cells influences remodeling of blood vessels in the lungs, which in turn leads to lung fibrosis.

Proper angiogenesis depends on the balance between pro- and anti-angiogenic factors. So, there are research works aiming at explanation of these process disturbances in the course of scleroderma and other connective tissue diseases. Correlation between endostatin levels and other factors is still not fully elucidated. VEGF exert its action on the release of different collagenases and other substances, which cleave endostatin from collagen XVIII, from endothelial cells [46, 47]. Increased endostatin levels were found in rheumatoid arthritis [48] and systemic lupus erythematosus [20]. Hebbar et al. observed some correlation between serum endostatin levels and cutaneous ulcers or scars developed in SSc patients [4]. Other authors however, have only found increased serum endostatin levels in a few patients without any correlation with clinical parameters [3]. Differences in the observed results are not clarified.

Our results demonstrated statistically significantly higher serum endostatin levels in SSc patients than in the control group. There were some differences in endostatin levels between limited and diffuse SSc patients, however they did not reach statistical significance. There was a strong but statistically insignificant, correlation between disease duration and skin fibrosis severity and increased serum endostatin levels. Lack of statistical significance could be the result of a relatively small number of the examined patients. Hebbar et al [4] and Hunzelmann et al [49] observed that in systemic sclerosis patients endostatin serum levels were increased and correlated with more extensive skin involvement.

In the course of scleroderma, collagen XVIII can be detected in the perivascular basement membrane zone of the skin and many internal organs involved [50]. Higher endostatin levels were observed in the examined SSc patients with the gastrointestinal tract, heart, lungs and osteo-articular systems involvement. However, only correlation between endostatin levels and cardiovascular system disturbances and pulmonary dysfunction found on spirometry, have reached statistical significance. It could be assumed that despite not full elucidation of endostatin action, this protein can exert an inhibitory effect on pro-enzymatic metalloproteinase 2. It can also inhibit catalytic action on membrane metalloproteinases type 1 and 2 [3]. In this way, endostatin can favor excessive deposition of extracellular matrix components and fibrosis development in the internal organs [30]. Hebbar et al also found that some patients with pulmonary fibrosis, which was detectable on chest radiograms, had higher endostatin concentrations [4]. These authors suggest that endostatin detected is released by tissue-activated fibroblasts.

Table 5. Serum levels of vascular endothelial growth factor (VEGF) (pg/ml) and endostatin (ng/ml) in systemic sclerosis patients with or without selected organ involvement

Internal organ			Vascular endothelial growth factor			Endostatin		
			Systemic Sclerosis	limited SSc	diffuse SSc	Systemic Sclerosis	limited SSc	diffuse SSc
oesophagus	–	$\bar{\chi}$±SD	189.1±163.3	216.9±222.5	152.1±46.5	97.6±19.6	96.5±21.2	99.1±21.6
		Me	153.7	177.1	153.7	102.0	96.4	108.0
		Range	0-513.3	0-513.3	104.8-197.7	71.2-122.0	71.2-122.0	74.4-114.8
	+	$\bar{\chi}$±SD	195.4±207.8	220.1±237.6	161.6±163.5	118.5±68.1	103.6±60.9	134.8±74.7
		Me	126.5	138.2	114.8	114	102.2	126.0
		Range	0-836.4	0-836.4	0-455.6	13.6-261.2	13.6-209.6	18.0-261.2
	p-value		>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
heart	–	$\bar{\chi}$±SD	154.8±120.7	145.8±98.0	168.9±157.7	86.3±50.2	67.4±34.2	108.0±59.1
		Me	143.5	138.2	153.7	84.4	77.8	114.8
		Range	0-455.6	0-326.6	0-455.6	13.6-194.4	13.6-103.6	18.0-194.4
	+	$\bar{\chi}$±SD	241.1±257.9	320.7±316.5	150.2±142.2	140.9±59.3	136.3±46.5	146.2±74.9
		Me	114.8	267.5	114.8	123.6	118.0	124.8
		Range	0-836.4	0-836.4	0-404.9	46.4-261.2	82.0-209.6	46.4-261.2
	p-value		>0.05	>0.05	>0.05	0.01*	0.0006*	>0.05
joint	–	$\bar{\chi}$±SD	190.0±144.0	132.7±78.7	304.7±213.5	81.4±13.8	87.6±15.1	72.0±3.4
		Me	129.3	102.4	304.7	74.4	90.8	72.0
		Range	76.5-455.6	76.5-249.3	153.7-455.6	69.6-100.8	71.2-108.0	69.6-74.4
	+	$\bar{\chi}$±SD	195.0±209.0	242.6±251.0	135.4±126.9	120.1±64.5	105.1±58.5	136.3±69.2
		Me	138.2	148.7	109.8	114.8	103.6	125.4
		Range	0-836.4	0-836.4	0-404.9	13.6-261.2	13.6-261.2	18.0-261.2
	p-value		>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Chest X-ray	–	$\bar{\chi}$±SD	132.4±132.9	111.8±96.5	173.6±189.1	117.2±63.0	104.0±54.8	138.1±73.6
		Me	100.0	95.2	114.8	102.8	100.8	123.6
		Range	0-455.6	0-326.6	0-455.6	32.4-261.2	32.4-209.6	46.4-261.2
	+	$\bar{\chi}$±SD	302.0±246.1	521.0±224.1	145.6±95.1	108.8±59.3	97.2±55.0	116.2±65.3
		Me	216.8	513.3	153.7	115.0	106.4	124.8
		Range	0-836.4	219.9-836.4	0-269.9	13.6-233.6	13.6-162.0	18.0-233.6
	p-value		0.02*	0.002*	>0.05	>0.05	>0.05	>0.05
spirometry	–	$\bar{\chi}$±SD	148.5±135.8	137.3±106.0	173.8±206.2	106.2±70.6	70.3±28.0	160.0±84.9
		Me	104.8	104.8	119.9	87.6	77.8	154.6
		Range	0-455.6	0-326.6	0-455.6	32.4-261.2	32.4-102.0	69.6-261.2
	+	$\bar{\chi}$±SD	223.6±226.4	293.4±285.5	153.9±126.0	117.4±56.2	120.8±56.6	114.0±59.7
		Me	151.2	184.3	134.3	118.0	110.2	124.2
		Range	0-836.4	0-836.4	0-404.9	13.6-233.6	13.6-209.6	18.0-233.6
	p-value		>0.05	>0.05	>0.05	>0.05	0.03*	>0.05

– normal; + abnormal
**statistically significant difference – comparison between group with or without selected organ involvement*
$\bar{\chi}$±SD – mean value±standard deviation

Quite interesting is another observation i. e. the lower serum VEGF levels the higher serum endostatin levels.

Our research showed that disturbances in levels of pro- and antiangiogenic factors play an important role in pathogenesis of systemic sclerosis and their serum levels can correlate with lung and cardiovascular system involvement.

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