Circulating endothelial progenitor cells in systemic sclerosis: relation to impaired angiogenesis and cardiovascular manifestations

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Abstract

Objective

Given the essential role of endothelial progenitor cells (EPCs) in endothelial repair and neovascularization, it is likely that insufficient angiogenesis seen in systemic sclerosis (SSc) is related to EPC alterations. The present study was aimed to analyze in SSc the number of circulating EPCs and their contribution into cardiovascular involvement.

Methods

EPC (CD34+VEGF-R2+ and CD133+VEGF-R2+) circulating levels were evaluated in 40 SSc patients and 24 controls by FACS; their correlations with peripheral vascular manifestations, heart involvement, Framingham risk score, carotid artery disease, endothelial function and morphological signs of microangiopathy were studied.

Results

Early stage SSc and high disease activity were accompanied by a rise in circulating EPC levels in association with increased membrane expression of Fas (CD95) that correlated positively with severity of peripheral vascular manifestations. EPC reduction with disease progression was linked with endothelial dysfunction and capillary loss, and showed a strong relation to the development of severe internal organ (predominantly cardiac) involvement and pulmonary hypertension. There was a tendency to decreased EPC levels in SSc pts with low HDL values, but no significant correlations were found between EPCs and Framingham risk factor score, carotid artery IMT and traditional cardiovascular risk factors.

Conclusions

In early stage SSc mobilization of EPCs in response to tissue ischemia was preserved, but dropped with disease progression. EPC reduction may contribute to endothelial dysfunction and impaired angiogenesis, leading to the development of severe vascular life-threatening complications of SSc. Traditional cardiovascular risk factors and subclinical atherosclerosis did not influence EPC levels in SSc patients.

Key words

Endothelial progenitor cells, systemic sclerosis, angiogenesis, endothelial dysfunction, cardiovascular disease.

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Introduction

Systemic sclerosis (SSc) is a connective tissue disease characterized by cutaneous and visceral fibrosis (1), and widespread vascular pathology (2). The underlying cause for SSc remains poorly understood, but it is well established that microvascular endothelial cells are the target tissue in this disease (3). A sequential study of the pathological changes in SSc patients revealed functional and structural endothelial aberration as the first anomaly, followed by lymphocyte migration and tissue fibrosis (4). However, despite widespread endothelial damage, blood occlusion and reduced tissue oxygenation, the process of neovascularization aimed to re-establish blood flow appears to fail in SSc, probably due to the altered capacity of endothelial cells to repair.

Endothelial progenitor cells (EPCs) are known to constitute key cellular effectors of vascular regeneration. They represent a heterogeneous cell population originated from a single multipotent progenitor within the bone marrow (BM) and consist of cells at different stages of maturation – ranging from early CD133+/VEGF-R2+ to more mature CD34+/VEGF-R2+ phenotypes (5-7). In spite of the phenotypic differences, these cells share the ability to differentiate towards mature endothelial cells and incorporate into the injured vasculature, thereby promoting neovascularization and subsequent functional recovery of the surrounding tissue (8, 6, 9). A variety of results suggest that impaired BM mobilization or depletion of these cells contribute to endothelial dysfunction and progression of vascular diseases (10-13). Thus, hypothetically, the devastated endothelial state and the lack of sufficient angiogenesis seen in SSc patients might be explained by modifications of circulating EPC numbers.

Recently published data on EPC quantitative and functional characteristics in SSc are inconsistent and apparently conflict with each others: EPC levels were reported to be higher (14, 17) and lower (16) compared with healthy controls, the correlation with disease duration found by Del Papa *et al*. was not confirmed by the others (16). The same contradictions concern EPC link with clinical picture of SSc (14,16). The relationships between EPC alterations and endothelial dysfunction, impaired angiogenesis and severity of vascular SSc manifestations as well as their contribution into internal organ damage remain unknown. The latter issue seems particularly important in view of the fact that endothelial injury underlies the development of kidney, cardiac and pulmonary disease associated with a high mortality rate in SSc (18).

To clarify these questions we assessed the numbers of circulating EPCs at different stages of differentiation in SSc patients with early and late disease putting an emphasize on the detailed analyses of peripheral vascular involvement, internal organ damage, quantitative characteristics of capillary abnormalities and endothelial function. EPC contribution into vascular manifestations was separately studied in early and late SSc. Considering the fact that SSc associates with increased cardiovascular mortality, we also analyzed the prevalence of carotid artery disease in SSc patients, the influence of traditional cardiovascular risk factors on the numbers of EPC in blood and their impact on SSc macrovascular disease.

Patients and methods

Patients.

Forty consecutive patients with SSc (all female) aged from 19 to 60 years (mean±SD = 44±11 yrs) were recruited in the State Institute of Rheumatology (Moscow). Patients were classified as affected by lcSSc (n=25; 62%) or dcSSc (n=15; 38%) disease according to LeRoy *et al*. (19). The duration of disease varied from 2 months to 25 years (6.4±6.2 yrs). Twenty patients were in an early stage of SSc as defined by Medsger and Steen (20). Exclusion criteria: overlap syndromes, juvenile SSc, malignancies, inflammatory disease and the history of major cardiovascular events.

Clinical assessment.

Characteristics of SSc patients are summarized in Table I. Skin involvement was assessed with the modified Rodnan skin score (21). Disease activity was determined by measuring SSc activity score (22). 28% of SSc patients had

Table I. Clinical characteristics of the SSc patients.

		SSc patients (n=40)
Age, mean ± SD years		44,3 ± 10.9
Disease duration, mean ± SD years	from RP	7.6 ± 7.1
	from first non-RP feature	6.4 ± 6.2
SSc subset, %	dcSSc / lcSSc	38/62
Disease activity, %	active / inactive	28/72
Disease severity, %	I / II	55/45
Weight loss, %		35
Raynaud's phenomenon, %		100
Fingertip ulcers and/or pitting scars, %		40
Skin score, mean±SD		11±10
Teleangectasia, %		38
Hyperpigmentation, %		43
Tendon friction rubs, %		13
Contractures, %		70
Calcinosis, %		22
Arthritis/tenosynovitis, %		38
Muscle weakness, %		15
Visceral involvement, %: Lung		93
Heart		35
Gastrointestinal		95
Kidney		3
Severity of pulmonary disease, %	0/1/2/3/4	7/40/23/30/0
Severity of cardiac disease, %	0/1/2/3/4	65/17.5/17.5/0
Severity of gastrointestinal disease, %	0/1/2/3/4	5/87/8/0/0
Severity of kidney disease, %	0/1/2/3/4	97/3/0/0/0
Esophageal involvement, %		80
Gastric involvement, %		32
Intestinal involvement, %		12
Conduction defects, %		23
Arrhythmia, %		20
Pericarditis, %		28
HRCT findings of interstitial lung disease, %		90
Restrictive abnormalities, %		48
FVC<80%, % of patients		25
DLCO<80%, % of patients		70
Pulmonary hypertension, %		28
a-Scl-70+/ACA+, %		60/12.5

Table II. Peripheral vascular manifestations in the SSc patients (n=40).

Measurement		
Progression of vascular disease by the time of investigation, % pts (no.)		35% (14)
Fingertip ulcers, % pts (no.)		28% (11)
No. of ulcers per patient, mean±SD (range)		2.7 ± 2 (1-8)
No. of ulcers per year, mean±SD (range)		6 ± 4.8 (1-16)
Pitting scars, % pts (no.)		40% (16)
Raynaud's Condition Score, mean ± SD (range)		2.8 ± 2.6 (0-8)
Patient's assessment of RP (by VAS), mean ± SD (range)		45 ± 26 (0-100)
Patient's assessment of digital ulcers (by VAS), mean ± SD (range)		16 ± 31 (0-100)
Physician's assessment of RP (by VAS), mean ± SD (range)		38 ± 21 (0-100)
Physician's assessment of digital ulcers (by VAS), mean ± SD (range)		28 ± 35 (0-100)
SHAQ VAS for pain, mean ± SD (range)		0.97 ± 0.96 (0-3)
SHAQ VAS for RP, mean ± SD (range)		1.2 ± 0.98 (0-3)
SHAQ VAS for digital ulcers, mean ± SD (range)		0.74 ± 1.19 (0-3)
Severity of Raynaud's phenomenon, % pts	0/1/2/3/4 grade	0/55/17/28/0

active (≥3) and 78% – inactive disease. Severity of organ involvement was determined as suggested by Medsger (23) with subsequent subgrouping SSc patients by severity of internal organ damage:

I grade of severity (55% of SSc pts) – mild visceral organ involvement: pulmonary (bilateral interstitial pulmonary fibrosis on chest roentgenogram, reduction of FVC ≥70%) or/and GI (distal esophageal hypoperistalsis) or/and cardiac (ECG conduction defect, LVEF ≥45%) or/and renal (serum creatinine ≤ 1.6 mg/dl, urine protein <1500mg/24 hours, creatinine clearance ≥60ml/min).

II grade of severity (45% of SSc pts) – severe visceral organ involvement: pulmonary (FVC <70% of predicted value or the presence of pulmonary hypertension) or/and GI (malabsorption syndrome or episodes of pseudo-obstruction) or/and cardiac (arrhythmia, LVEF <45%) or/and kidney (serum creatinine >1.6 mg/dl, urine protein ≥1500mg/24 hours, creatinine clearance <60ml/min).

All patients completed the Health Assessment Questionnaire (HAQ) including a visual analog scale (VAS) for pain and 5 scleroderma-specific VAS scales (SHAQ VAS) developed by Steen and Medsger (24) to measure the impact of SSc on the daily activity of intestinal disease, breathing problems, RP, digital ulcers, and overall disease during the previous week. These 15-cm scales were scored from 0 to 3. Both the patients and the physicians also used 15-cm VAS scales (anchored 0-100) to rate the activity of Raynaud's phenomenon (RP) and digital ulcers (25). Severity of peripheral vascular disease was assessed using the scale originally developed by Medsger (23) The number of cutaneous ulcers at the time of blood drawing, progression of vascular disease within the last month, and the number of new ulcers in the past year were recorded by physicians (Table II). All patients completed the Raynaud's Condition Score (RCS) – a daily self-assessment of RP activity using a 0-10 ordinal scale that incorporates the cumulative daily frequency, duration, severity, and impact of RP attacks (24).

At the time of the study, twelve (30%) patients were not receiving any potentially disease-modifying drugs. Concomitant treatment of the others included corticosteroids ≤10mg/day (28 pts, 70%), D-penicillamine (12 pts, 30%), cyclophosphamide (8 pts, 20%) and methotrexate (3 pts, 7%). No vasoactive medications were taken for at least one week before the study. Iloprost and Statins were never used in these patients.

Twenty-four healthy volunteers who had neither a history of ischemic cardiovascular events nor any inflammatory or autoimmune diseases were selected to be matched for age (45±9yr, p=0.5) and sex (all female), and served as controls.

Nailfold videocapillaroscopy.
Microvascular pathology was studied quantitatively in 30 SSc pts and 20 controls using nailfold video capillaroscopy (NVC). The nailfold capillaries of digits 2-5 bilaterally were examined by a videomicroscope (Leica MZ6, DCC "Sony" DXC 107AP) in 20x and 800x magnifications. The *quantitative assessment* of nailfold capillary abnormalities included the measurement of capillary density (loops/mm of the distal row), the mean capillary width (μm) and the mean capillary length (μm). The percentage of enlarged and bushy loops were measured as the mean number of these loops of the total number of capillaries/mm. in the distal row. Nailfold bleeding/finger was classified as grade 1 (punctuate haemorrhage ≤2/finger), grade 2 (punctuate haemorrhage >2/finger), grade 3 (confluent areas of haemorrhage). *Semiquantitatively* we assessed the number of avascular areas (0 = absence, 1 = ≤2 discrete areas of vascular deletion, 2 = > 2 discrete areas of vascular deletion, 3 = the presence of large, confluent avascular areas) and their size (1 = deletion of 1-3 capillaries, 2 = deletion of 4-6 capillaries, 3 = deletion of more than 6 capillaries). In general, avascularity of the capillary bed was measured as the "number x size" of avascular areas. *Qualitative evaluation* was performed of the entire distal row of capillary loops and included the assessment of pattern (26), presence of extravasates and visibility of loops: 1 = partial or total loops, 2 (significant perivascular edema) = only tops of the loops.

Assessment of endothelium-dependent and endothelium-independent function
Imaging of the brachial artery proximal to the antecubital fossa was performed with the use of high-resolution ultrasonography. Endothelium-dependent flow mediated vasodilatation was assessed by measuring the maximal increase in the diameter of the brachial artery during reactive hyperemia evoked by the release of a cuff inflated to 225 mm Hg for five minutes on the upper arm, proximal to the measurement site. After a rest period of 15 minutes, base-line measurement of diameter was repeated, and 0.4 mg of nitroglycerin spray was administered sublingually to assess endothelium-independent vasodilatation.

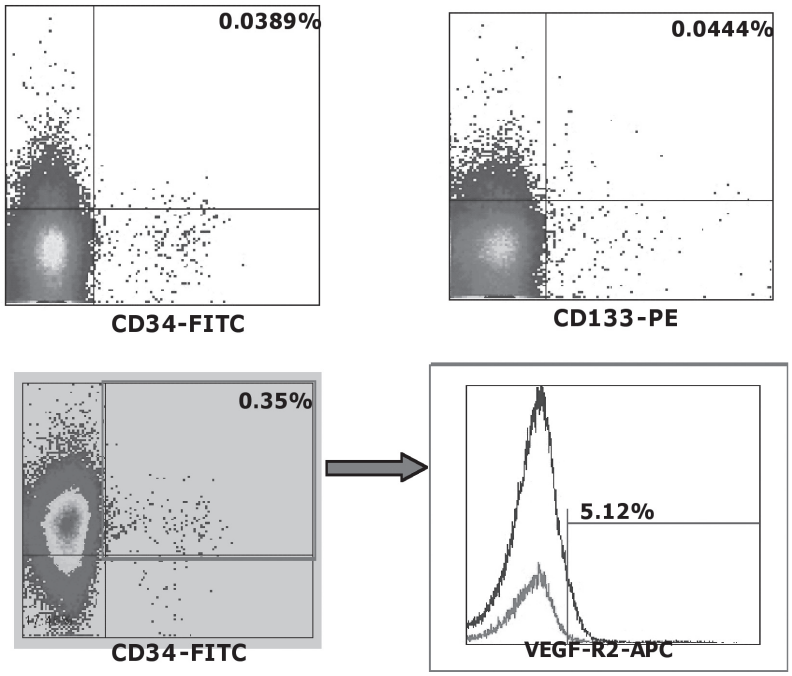

Fig.1. Co-expression of CD34/VEGF-R2, CD133/VEGF-R2 and CD95/CD34 surface markers on the peripheral blood cells of SSc patient.

Subclinical atherosclerosis detection
Duplex ultrasonography was performed to assess the carotid artery intima-media thickness (IMT) nd plaque. A total of three IMT measurements on each side were taken at the following points: common carotid artery (10 mm before the bulb), bulb (5-10 mm cranially to the start of the bulb), and internal carotid arteries (15 mm after the flow divider). Mean IMT (m-IMT; the mean of the three IMT measurements on each side) and the maximum IMT (M-IMT, the highest IMT value found among the six segments studied) were assayed. According to current sonographic criteria, we referred to "normal" IMT when complex intima-media was ≤0.9 mm. M-IMT values >0.9 mm were considered indicative of thickened intima and M-IMT values >1.3 mm indicative of atherosclerotic plaque.

Flow cytometry analysis
The numbers of circulating EPCs were detected by flow cytometry method that is commonly used to measure EPC levels in peripheral blood (27) and validated in several studies (28, 29). In brief, 100 μL of peripheral blood was incubated with allophycocyanine (APC)-conjugated monoclonal antibody against human VEGF-R2 (R & D Systems), followed by staining with both fluorescein isothiocyanate (FITC)-conjugated CD34 (Immunotech) and/or phycoerythrin-conjugated AC133 (Miltenyi Biotec) antibodies. CD34/VEGF-R2 and CD133/ VEGF-R2 double-positive cells within the lymphocyte population were characterized as EPCs (Fig 1). Control staining was performed with isotype-matched antibodies. Incubation was followed by lysis and fixation with Human Erythrocyte Lysing Kit (R&D Systems). The analysis was made by FACSCalibur (Becton Dickinson) in 40 patients and 24 controls. Acquisition performed up to $5x10^5$ events per sample. CD34+/ VEGF-R2+ cells were studied additionally for CD95-positivity using phycoerythrin-conjugated CD95 (Fas) antibodies (Beckman Coulter) in 19 SSc patients and 16 controls.

Table III. Range variations and median±SE for progenitor cells in peripheral blood of the SSc patients and controls.

	SSc patients (n=40)		Controls (n=24)		*p*
	median ± SE	range	median ± SE	range	
CD34+VEGF-R2+	0.0080 ± 0.0024	0.0001-0.0920.0	037 ± 0.0005	0.0001-0.0098	< 0.01
CD133+VEGF-R2+	0.0159 ± 0.0026	0.0001-0.075	0.0102 ± 0.0016	0.0015-0.0334	< 0.05
CD34+CD95+*	0.077 ± 0.021	0.008-0.356	0.036 ± 0.006	0.013-0.105	< 0.005
VEGF-R2+CD95+*	0.41 ± 0.06	0.006-1.12	0.22 ± 0.05	0.14-0.89	< 0.005
CD34+VEGF-R2+CD95+*	0.0076 ± 0.0025	0.0001-0.0464	0.0038 ± 0.0006	0.0001-0.0080	< 0.005

*Data for 19 SSc patients and 16 controls.

Statistical evaluation

All data are presented as mean ± SEM, unless stated otherwise. Statistical evaluations were performed with SPSS for Windows, version 6.0 (SPSS Inc). Nonparametric tests (Mann-Whitney U, Kruskal-Wallis H, and Spearman correlation) were used when appropriate. A probability value <0.05 was considered statistically significant.

Results

EPC numbers in blood of SSc patients were elevated compared to controls; most CD34/VEGF-R2-positive cells demonstrated membrane expression of Fas (CD95).

Range variations and median±SE for progenitor cells in the whole study samples are shown in Table III. In SSc both early CD133+/VEGF-R2+ and more mature CD34/VEGF-R2-positive cell populations were significantly increased. Most CD34+/VEGF-R2+ EPCs expressed membrane CD95 (0.0076±0.0025% in SSc patients *vs*. 0.0038±0.0006% in controls, $p<0.005$).

Early stage of SSc and high disease activity were accompanied by a rise in EPC levels. Late SSc patients and those with severe internal organ (predominantly cardiac) involvement and/or pulmonary hypertension showed lower numbers of circulating EPCs.

The frequency of circulating EPCs was significantly higher in early SSc compared to advanced stage (0.02±0.01% *vs*. 0.006±0.005%, $p<0.01$). In fact, EPC levels in peripheral blood were inversely correlated with disease duration as assessed from both the onset of Raynaud's phenomenon (r=-0.45, p=0.0037) and from the first non-Raynaud's symptom (r=-0.47, p=0.002) (Fig. 2).

SSc patients with active disease (≥3) showed slightly higher EPC levels compared with inactive-SSc patients or SSc patients with low disease activity (0.02±0.024 *vs*. 0.009±0.008, $p<0.05$). Severe visceral pathology was associated with reduced numbers of EPCs in blood (0.007±0.005 *vs*. 0.01±0.009, $p<0.05$). When organ involvement was analyzed further, EPC levels were found to inversely correlate with severe cardiac disease (Table IV) and were decreased in patients with pulmonary hypertension defined as echocardiographic signs of systolic pulmonary arterial pressure >35 mmHg confirmed on right heart catheterization (0.005±0.004 *vs*. 0.01±0.008, $p<0.05$). No correlations were found between the numbers of EPCs and SSc subset (r= -0.15, p=0.36), skin score (r=-0,17, p=0,3) and HAQ/SHAQ (r=-0.01, p=0.9/r= -0.27, p=0.24).

Severe peripheral vascular manifestations were associated with increased numbers of circulating EPCs, the highest cell levels were found in early SSc patients with digital ulcers. There was a lack of such EPC mobilization from BM in response to severe ischemia and tissue damage in the late stage of disease.

SSc patients (n=21) with digital pit ulceration or/and elevated Raynaud's Condition Score (>3) showed higher levels of CD34+/VEGF-R2+ EPCs (0.02±0.006 *vs*. 0.01±0.007, $p<0.05$). There was a positive correlation between EPCs and VAS for RP activity (r=0.34, $p<0.05$). Subanalyses performed of early-SSc and late-SSc groups revealed that EPC mobilization in response to ischemic ulcer formation

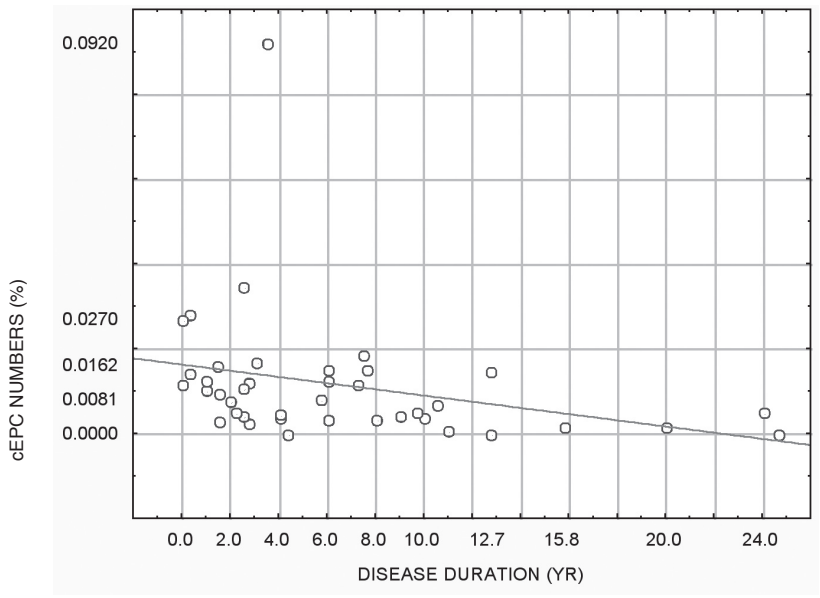

Fig. 2. Inverse correlation between the circulating EPC numbers (CD34+/VEGF-R2+) and SSc duration (n=40, Spearman's rank correlation).

Table IV. Correlation between EPC numbers (CD34+/VEGF-R2+) in blood and severity of organ involvement in the SSc patients (n=40, Spearman's rank correlation).

Severity of organ involvement	*r*	*p*
Skin (grade 0-4)	0.06	0.7
Joint/tendon (grade 0-4)	-0.17	0.3
Muscle (grade 0-4)	0.23	0.2
Lung (grade 0-4)	-0.09	0.6
Heart (grade 0-4)	**-0.38**	**0.01**
Gastrointestinal tract (grade 0-4)	-0.21	0.2
Kidney (grade 0-4)	0.11	0.47

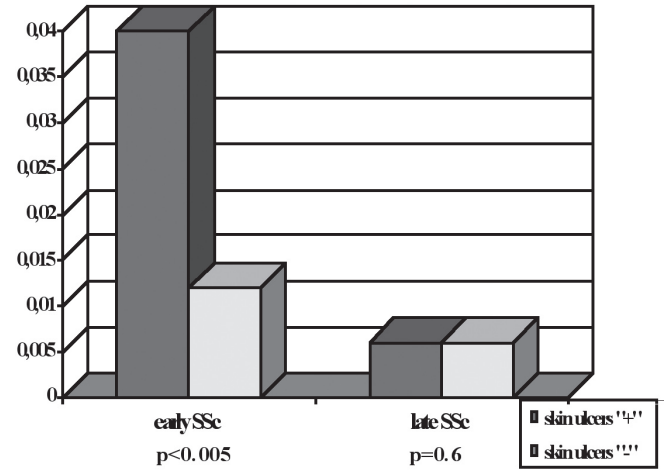

Fig. 3. Association between the elevated EPC levels in blood and fingertip ulcers in patients with early SSc (n=20) and the lack of EPC mobilization in response to tissue ischemia in late SSc (n=20)

Table V. Nailfold capillary abnormalities in the SSc patients compared with healthy controls and their correlations with circulating EPC levels (Spearman's rank correlation).

Capillary dimensions		SSc patients (n = 30)	Controls (n = 24)	Spearman's rank correlation
Capillary density, loops/mm		5.48±1.8***	8.7±0.9	*r*=0.4, *p*<0.05
Mean capillary width (μm)		47.33±16.2***	20.7±4.4	NS
% of enlarged capillaries		64.22±22.04***	4.25±2.8	NS
% of changed capillaries		75.9±21.3***	9.35±5.7	*r*=-0.43, *p*<0.01
% of bushy capillaries		27.4±22.2***	0.1±0.2	NS
Perivascular edema, %		40**	0	NS
Hemorrhage (presence), %		50**	7	NS
Hemorrhage (grade), %	1	26*	7	NS
	2	7	0	
	3	17**	0	
Pattern, %	Early	30	-	NS
	Active	57	-	
	Late	13	-	

*p<0,05, **p<0,005, ***p<0,0005

was characteristic only for early SSc patients (Fig 3).

EPC reduction in blood was associated with endothelial dysfunction and morphological signs of destructive microangiopathy.

Measurement of flow-mediated brachial-artery reactivity in 30 SSc patients revealed that in cases with lowered endothelium-dependent vasodilatation (change from base line of ≤10%) the numbers of CD34/VEGF-R2-positive cells were significantly lower than in patients with preserved endothelial function (0.006±0.004% *vs*. 0.02±0.018%, *p*=0.03). No correlation was found between EPCs and endothelium-independent vasodilatation (change from base line in SSc group: 31.1±20.4%; *r*=0.002, *p*>0.5). The analysis of capillary changes revealed a positive link between cEPC numbers and capillary density (*r*=0.4, *p*<0.05) as well as an inverse correlation with the percentage of abnormally changed capillaries (*r*=0.43, *p*=0.008) (Table V, Fig. 4).

Traditional cardiovascular risk factors did not influence circulating EPC levels in SSc. No connection was found between EPC numbers in peripheral blood and subclinical atherosclerosis.

SSc patients showed a high prevalence of carotid artery disease: thickened media (M-IMT values >0.9 mm) and atherosclerotic plaques (M-IMT values >1.3 mm) were revealed in 33% (13/40) and 5% (2/40) of pts, consequently, predominantly in rapidly progressive disease with severe organ damage (*r*=0.33, *p*<0.05) and late stage SSc (*r*=0.55, *p*<0.001). High Framingham coronary risk prediction (≥20) was found in 2% of SSc pts, intermediate (5-20) in 38%, low (<5) in 60%. The prevalence of traditional cardiovascular (CV) risk factors in a study group of SSc patients is shown in Table VI. Carotid artery IMT was positively linked with Framingham risk factor score (*r*=0.44, *p*<0.01), age (*r*=0.36, *p*<0.05), postmenopausal state (*r*=0.43, *p*<0.01) and systolic blood pressure (*r*=0.39, *p*<0.01). There was a tendency to decreased EPC levels in SSc pts with low HDL values (*p*=0.07), but no significant correlations were found between EPC and Framingham risk factor score, carotid artery IMT, hsCRP and traditional CV risk factors.

Discussion

Our data demonstrate an increase of circulating EPCs in early stage of systemic sclerosis that correlated positively with the severity of Raynaud's phenomenon and the presence of digital ulcers. This prompts the hypothesis that EPCs are mobilized from BM in response to microvascular injury seen in early SSc. In fact, tissue ischemia is a stimulus for production of VEGF, SDF-1 and other angiogenic factors (30), which influence EPC mobilization (31). This mechanism seems to be preserved in early SSc, leading to an increased number of circulating EPCs. Consistent with our data are findings on the elevated levels of VEGF in blood and skin of SSc patients (32, 33). The

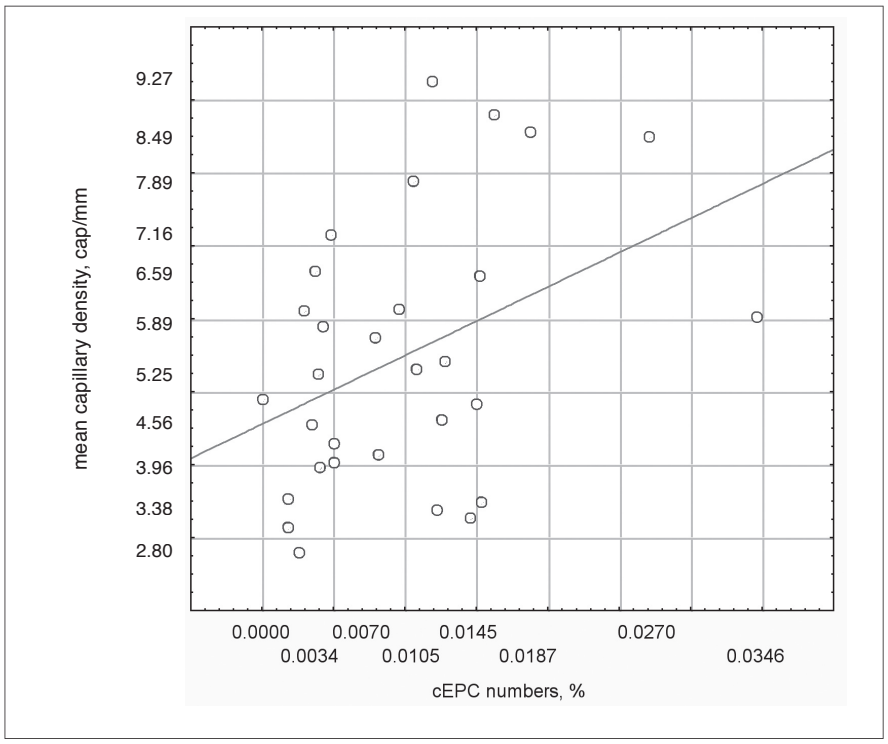

Fig. 4. Positive correlation between the circulating EPC numbers (CD34+/VEGF-R2+) and capillary density (cap/mm) in the SSc patients (n=30).

Table VI. The prevalence of traditional cardiovascular risk factors in the SSc patients (n=40).

Characteristics	n	(%)
Total cholesterol ≥200 mg/dl	11	(27.5)
HDL cholesterol <50 mg/dl	12	(30)
Systolic blood pressure ≥140 mm Hg	4	(10)
Current smokers	7	(17.5)
Diabetes mellitus	2	(5)
BMI>25 kg/m^2	9	(22.5)
Sedentary lifestyle	10	(25)
Family history of premature atherosclerotic CVD events	11	(27.5)

highest EPC numbers found in early SSc patients with digital ulcers also confirm the normal supply of progenitor cells. Thus, early active SSc was characterized by an increased drive towards vascular regeneration with an adequate response of BM stem cells.

However, despite the increased EPC circulating levels, most of the cells showed surface expression of Fas (CD95), suggestive of a pre-determined commitment to programmed cell death. In SSc, antibody-mediated endothelial apoptosis with induction of the Fas pathway is known as a crucial pathogenetic event (34). Since EPCs share many structural and functional properties of mature endothelial cells (ECs), they are likely exposed to the same stimuli that cause EC apoptosis in SSc patients such as anti-hCMV peptide antibodies or/and antiendothelial cell antibodies known to induce apoptosis through antibody-dependent cell-mediated cytotoxicity via Fas (35, 36). Therefore, the negative influence of multiple stimuli, resulting in the induction of an apoptotic response at EPC level, may provide an impediment to a proper angiogenic process in SSc.

The increased EPC turnover due to apoptosis and/or intrinsic cell abnormalities (15) could partly explain the discrepancy between the elevated numbers of EPCs and the impaired ulcer healing seen in SSc patients. However, further works on EPC tissue levels and cytokine milieu of the local environment in SSc ischemic ulcers are needed to clarify this issue. It has been paradoxically shown that in spite of preserved EPC mobilization from BM, granulation tissue in ischemic skin lower-extremity wounds contains much lower numbers of EPCs as compared to non-ischemic ones (37). These findings suggest that the reduction in EPC response on tissue level contributes to the delayed closure of ischemic defect. In such cases the direct injection of EPCs to repopulate the local wound pool of progenitor cells within granulation tissue or/and the local use of stimulatory cytokines known to influence EPC mobilization may have better chances of a therapeutic benefit than systemic transfusion of progenitor cells.

While the disease advanced, EPC levels significantly dropped with the lack of EPC mobilization from BM in response to digital ulcer formation and severe tissue hypoxia in the late stage of SSc. In general, there was found an inverse correlation between disease duration and the numbers of circulating EPCs that is in line with the findings of Del Papa *et al.* (14). Hence, the continuous endothelial damage in SSc likely leads to the exhaustion of the stem cell pool. The altered production of angiogenic factors in late SSc (32) may be another reason for EPC reduced levels. The contradictory results on EPC levels in published papers can be partly explained by different cell phenotypes analyzed (14, 16, 17) due to the lack of clear EPC definition as well as relatively small study cohort (16) or some peculiarities of spontaneous consecutive presenting group (predominantly late SSc with the mean disease duration of 10 years in the work of Kuwana *et al.*).

Our study showed an association of EPC reduction in blood with pronounced functional and structural vessel changes in SSc patients. cEPC levels were found inversely linked with endothelium-dependant flow-mediated vasodilatation and nailfold capillary density. Thus, the exhaustion of EPC numbers in advanced disease following the chronic vascular damage, may contribute to the impaired endothelial regeneration and the loss of microvessels characteristic for SSc. These findings

are in line with experimental evidence for an important role of EPCs in endothelial repair and neovascularization. The link between EPC reduction and endothelial dysfunction has been previously shown in cardiovascular diseases (12, 38). It is now well established that endothelial injury in relation to insufficient numbers of EPCs in blood may affect the progression of coronary artery disease and the level of EPCs is considered a surrogate biologic marker for cumulative cardiovascular risk (6, 11, 12). Similarly, we found a strong association between EPC reduction and severity of cardiac disease in SSc patients. It might be speculated that the low levels of EPCs contribute to the development of microangiopathy related to SSc cardiac involvement or/and premature atherosclerotic lesions of coronary arteries in patients at advanced stage of SSc with an exhaustion of progenitor pool in BM. The letter seems less likely taking into consideration our data on the lack of correlations between EPC circulating levels and subclinical atherosclerosis as well as a 10-year coronary Framingham risk. We also showed that traditional cardiovascular risk factors and elevated hsCRP levels did not influence circulating EPCs, but a tendency to decreased EPC numbers was found in SSc pts with low HDL cholesterol values. In line with our findings, Werner *et al*. demonstrated that HDL cholesterol directly correlated with EPC numbers in patients with coronary artery disease, indicating that at least part of its vasculoprotective action may be mediated by EPCs (27).

Endothelial dysfunction also represents the earliest alteration in the development of primary pulmonary hypertension (PH) and vascular remodeling found in secondary PH (39, 40). It is hypothesized that EPC depletion can aggravate endothelial dysfunction and disturbed vascular homeostasis in lungs leading to the progression of PH regardless of its nature. Accordingly, we have found that SSc patients who developed PH had lower EPC numbers in blood compared with those without PH. Fadini *et al*. also revealed EPC depletion in patients with restrictive lung disease prompted to secondary PH (10). An important role of these cells in the pathogenesis of hypoxia-induced PH has been confirmed by the experimental results of successful EPC therapy for this condition: intravenously injected EPCs were shown to home to pulmonary vasculature and ameliorate pulmonary vascular remodeling due to the direct cell incorporation into the pulmonary endothelial integrity (41, 42).

In conclusion, while blocking endothelial cell death must be the primary goal in early SSc, as long as large numbers of EPC are available, their replacement may be a useful therapeutic intervention in late stage disease. Such studies aimed to EPC use for the repair of damaged endothelium and initiating vasculogenesis are currently under intense investigation in a number of conditions accompanied by severe tissue ischemia (43) providing preliminary clinical evidence of feasibility, safety, and efficacy of EPC therapy (44, 45). However, the negative influence of multiple stimuli, resulting in the induction of an apoptotic response at the endothelial level, or/and intrinsic EPC abnormalities (15) may serve a potential limitation of a regenerative EPC therapy approach in SSc patients.

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References

1. STRELHOW D, KORN JH: Biology of the scleroderma fibroblast. *Curr Opin Rheumatol* 1998; 10: 572-78.
2. MATUCCI CERINIC M, KAHALEH BM, LEROY EC: The vascular involvement in systemic sclerosis. *In* FURST D, CLEMENTS P (Eds.) *Systemic sclerosis*. Philadelphia: Lea and Febiger, 1995; 513-74.
3. KAHALEH B: Lymphocyte interactions with the vascular endothelium in systemic sclerosis. *Clin Dermatol* 1994; 12: 361-67.
4. PRESCOTT RJ, FREEMONT AJ, JONES CJP *et al*.: Sequential dermal microvascular and perivascular changes in the development of scleroderma. *J Pathol* 1992; 166: 255-63.
5. GILL M, DIAS S, HATTORI K *et al*.: Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells. *Circ Res* 2001; 88: 167-74.
6. HRISTOV M, WEBER C: Endothelial progenitor cells: characterization, pathophysiology, and possible clinical relevance. *J Cell Mol Med* 2004; 8: 498-08.
7. RAFII S, LYDEN D: Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. *Nat Med* 2003; 9: 702-12.
8. ASAHARA T, MUROHARA T, SULLIVAN A *et al*.: Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997; 275: 964-67.
9. TAKAHASHI T, KALKA C, MASUDA H *et al*.: Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med* 1999; 5: 434-38.
10. FADINI G, SCHIAVON M, CANTINI M *et al*.: Circulating Progenitor Cells Are Reduced in Patients with Severe Lung Disease. *Stem Cells* 2006; 24:1806-13. Published online April 6, 2006.
11. GEORGE J, GOLDSTEIN E, ABASHIDZE S *et al*.: Circulating endothelial progenitor cells in patients with unstable angina: association with systemic inflammation. *Eur Heart J* 2004; 25: 1003-8.
12. HILL J, ZALOS G, HALCOX J *et al*.: Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 2003; 348: 593-600.
13. VALGIMIGLI M, RIGOLIN GM, FUCILI A *et al*.: CD34 and endothelial progenitor cells in patients with various degrees of congestive heart failure. *Circulation* 2004; 110: 1209-12.
14. DEL PAPA N, COLOMBO G, FRACCHIOLLA N *et al*.: Circulating Endothelial Cells as a Marker of Ongoing Vascular Disease in Systemic Sclerosis. *Arthritis Rheum* 2004; 50: 1296-1304.
15. DEL PAPA N, QUIRICI N, SOLIGO D *et al*.: Bone marrow progenitors are defective in systemic sclerosis. *Arthritis Rheum* 2006; 54: 2608-15.
16. KUWANA M, OKAZAKI Y, YASUOKA H *et al*.: Defective vasculogenesis in systemic sclerosis. *Lancet* 2004; 364: 603-10.
17. ALLANORE Y, BATTEUX F, AVOUAC J *et al*. Levels of circulating endothelial progenitor cells in systemic scleosis. *Clin Exp Rheumatol* 2007; 25: 60-6.
18. GUSEVA NG: *Systemic sclerosis in rheumatic diseases* (Ed. NASSONOV EL). Moscow: Medical Press 1997, 172-180.
19. LEROY EC, BLACK C, FLEISCHMAJER R *et al*.: Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. *J Rheumatol* 1988; 15: 202-5.

20. MEDSGER TA, STEEN VD: Classification, prognosis. *In* CLEMENTS PJ, FURST DE (Eds.) *Systemic sclerosis*. Baltimore, MD: Williams & Wilkins 1996, 51-79.
21. CLEMENTS P, LACHENBRUCH P, SEIBOLD J *et al*.: Inter and intraobserver variability of total skin thickness score (modified Rodnan TSS) in systemic sclerosis. *J Rheumatol* 1995; 22: 1281-85.
22. VALENTINI G, DELLA ROSSA A, BOMBARDIERI S *et al*.: European multicentre study of define disease activity variables and development of preliminary activity indexes. *Ann Rheum Dis* 2001; 60: 592-8.
23. MEDSGER TA, SILMAN AJ, STEEN VD *et al*.: A disease severity scale for systemic sclerosis: development and testing. *J Rheumatol* 1999; 26: 2159-67.
24. STEEN VD, MEDSGER TA JR: The value of the Health Assessment Questionnaire and special patient-generated scales to demonstrate change in systemic sclerosis patients over time. *Arthritis Rheum* 1997; 40: 1984-91.
25. MERKEL P, HERLYN K, MARTIN R *et al*.: Measuring disease activity and functional status in patients with scleroderma and Raynaud's phenomenon. *Arthritis Rheum* 2002; 46: 2410-20.
26. CUTOLO M, SULLI A, PIZZORNI C, ACCARDO S: Nailfold videocapillaroscopy assessment of microvascular damage in systemic sclerosis. *J Rheumatol* 2000; 27: 155-60.
27. WERNER N, NICKENIG G: Influence of cardiovascular risk factors on endothelial progenitor cells limitations for therapy? Arteriosclerosis, *Arterioscler Thromb Vasc Biol* 2006; 26: 257.
28. KONDO T, HAYASHI M, TAKESHIDA K *et al*.: Smoking cessation rapidly increases circulating progenitor cells in peripheral blood in chronic smokers. *Arterioscler Thromb Vasc Biol* 2004; 24: 1-6.
29. LAMBIASE PD, EDWARDS RJ, ANTHOPOULOS P *et al*.: Circulating humoral factors and endothelial progenitor cells in patients with differing coronary collateral support. *Circulation* 2004; 109: 2993-99.
30. LEUNG DW, CACHIANES G, KUANG W-J *et al*.: Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 1989; 246: 1306-9.
31. HATTORI K, DIAS S, HEISSIG B *et al*.: Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. *J Exp Med* 2001; 193: 1005-14.
32. DISTLER O, DEL ROSSO A, GIACOMELLI R *et al*.: Angiogenic and angiostatic factors in systemic sclerosis: increased levels of vascular endothelial growth factor are a feature of the earliest disease stages and are associated with the absence of fingertip ulcers. *Arthritis Res* 2002; 4: R11.
33. DISTLER O, DISTLER JH, SCHEID A *et al*.: Uncontrolled expression of vascular endothelial growth factor and its receptors leads to insufficient skin angiogenesis in patients with systemic sclerosis. *Circ Res* 2004; 95: 109-16.
34. SGONC R, GRUSCHWITZ MS, DIETRICH H *et al*.: Endothelial cell apoptosis is a primary pathogenetic event underlying skin lesions in avian and human scleroderma. *Clin Invest* 1996; 98: 785-92.
35. LUNARDI C, BASON G, CORROCHER R *et al*.: Induction of endothelial cell damage by hCMV molecular mimicry. *Trends Immunol* 2005; 26: 19-24.
36. SGONC R, GRUSCHWITZ MS, BOECK G *et al*.: Endothelial cell apoptosis in systemic sclerosis is induced by antibody-dependent cell-medoated cytotoxity via CD95. *Arthritis Rheum* 2000; 43: 2550-62.
37. BAUER S, GOLDSTEIN L, BAUER R *et al*.: The bone marrow-derived endothelial progenitor cell response is impaired in delayed wound healing from ischemia. *J Vasc Surg* 2006; 43: 134-41.
38. TAO J, JIN YF, YANG Z *et al*.: Reduced arterial elasticity is associated with endothelial dysfunction in persons of advancing age: comparative study of noninvasive pulse wave analysis and laser Doppler blood flow measurement. *Am J Hypertens* 2004; 17: 654-59.
39. BUDHIRAJA R, TUDER RM, HASSOUN PM: Endothelial dysfunction in pulmonary hypertension. *Circulation* 2004; 109: 159-65.
40. CELLA G, BELLOTTO F, TONA F *et al*.: Plasma markers of endothelial dysfunction in pulmonary hypertension. *Chest* 2001; 120: 1226-30.
41. SATO K, KAGAYA Y, NAKANO M *et al*.: Important role of endogenous erythropoietin system in recruitment of EPCs in hypoxia-induced pulmonary hypertension in mice. *Circulation* 2006; 113: 1442-50.
42. ZHAO Y, COURTMAN D, DENG Y *et al*.: Rescue of monocrotaline-induced pulmonary arterial hypertension using bone marrow-derived endothelium-like progenitor cells: efficacy of combined cell and eNOS gene therapy in established disease. *Circ Res* 2005; 96: 442-50.
43. ZAMMATETTI P, ZISCH AH: Adult 'endothelial progenitor cells' renewing vasculature. *Int J Biochem Cell Biol* 2005; 37: 493-503.
44. HUANG PP, LI SZ, HAN MZ *et al*.: Autologous transplantation of peripheral blood stem cells as an effective therapeutic approach for severe arteriosclerosis obliterans of lower extremities. *Thromb Haemost* 2004; 91: 606-9.
45. TATEISHI-YUYAMA E, MATSUBARA H, MUROHARA T *et al*.: Therapeutic Angiogenesis using Cell Transplantation (TACT) Study investigators. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. *Lancet* 2002; 360: 427-35.