CXCR4^{pos} circulating progenitor cells coexpressing monocytic and endothelial markers correlating with fibrotic clinical features are present in the peripheral blood of patients affected by systemic sclerosis

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ABSTRACT

There is still controversy regarding the role of circulating endothelial and progenitor cells (CECs/CEPs) in the pathogenesis of systemic sclerosis (SSc). Using a sequential Boolean gating strategy based on a 4-color flow cytometric protocol, an increased number of CD31^{pos}/CD184^{pos}(CXCR4)/CD34^{pos}/CD45^{pos} and CD31^{pos}/CD117^{pos} (c-kit-R) /CD34^{pos}/ CD45^{pos} hematopoietic circulating progenitor cells (HCPCs) was detected in SSc patients compared with healthy subjects. In SSc, no circulating mature and progenitor endothelial cells were observed, while an enhanced generation of erythroid progenitor cells was found to be correlated with the presence of CD117+ HCPCs. The presence of freshly detected CXCR4posHCPC was correlated either to the *in vitro* cultured spindle-shaped endothelial like cells (SELC) with an endo/myelomonocytic profile or to SDF-1 and VEGF serum level. These data are related to more fibrotic clinical features of the disease, thus supporting a possible role of these cells in fibrosis.

Key words: circulating hematopoietic progenitor cells, endothelial-like cells, monocytes, CXCR4, systemic sclerosis.

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Introduction

Recent studies demonstrated the presence of circulating EPCs and CEPs that have been shown to contribute to tissue regeneration and therapeutic vasculogenesis.[1-2] Circulating endothelial cells (CECs) are another cell population present in a wide variety of human inflammatory and pathological conditions (cancer, infectious and cardiovascular disease) suggesting a role for these cells as a biomarker of vascular disease.[3-5]

The recruitment of circulating progenitor cells for vascular repair seems to be correlated to the expression of specific *homing* receptors, such as *CXCR4* (CD184), and to the concentration of the stromal derived factor-1 (SDF-1)/CXCL12 (CXCR-4/SDF-1 axis) as their specific ligand.[6] Pro-angiogenetic factors (vascular endothelial growth factor-VEGF, erythropoietin, etc.) may also be involved in the mobilization process.[7] Systemic sclerosis (SSc) is an autoimmune disease of unknown etiology characterized by excessive fibrosis and microvascular abnormalities.[8] The presence of CECs and CEPs in this disease continues to be controversial. In fact, an enhanced generation of CEPs has been demonstrated in SSc[9] and high levels of CECs were found to be linked to the severity of pulmonary hypertension in its early stages.[10] Conversely, it has been suggested that a defective vasculogenesis related to the absence of CEPs/EPCs can explain vascular disease in SSc and in rheumatoid arthritis.[11,12]

Due to the fact that no standardized methods are used to define and detect these cells, the results are sometimes conflicting, and a functional and phenotypic overlap between hematopoietic and endothelial progenitor cells has often been

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a source of misunderstanding.[13,14] Since the methodological approach must be able to adequately redefine the nature of circulating progenitor cells, in this study we proposed a sequential gating multiparametric flow-cytometric analysis and progenitor cell replating assays with the aim of better characterizing and enumerating the circulating hematopoietic (HCPC) and/or endothelial progenitor cells in SSc. Serum levels of angiogenic cytokines, in particular the SDF-1, were correlated to the presence of either freshly detected or cultured circulating progenitor cells together with the clinical features in order to evaluate their possible role in SSc pathogenesis.

Design and Methods

Patients

Forty patients with SSc fulfilling the criteria for classification proposed by Le Roy *et al*.[15] and according to Medsger and Steen[8] were enrolled in this study (Table 1). Human peripheral blood (PB) samples were also obtained from 10 healthy subjects (NS) as controls (mean age 43±7, range 27-49; 3 males/7 females) after obtaining informed consent. All patients were evaluated for modified Rodnan skin score (mRSS) and lung high resolution computed tomography (HRCT) scored as reported by Warrick *et al*.[16] This study was carried out with the approval of the local ethics committee.

Flow cytometric analysis of circulating progenitor cell subsets from PB

A 4-color cytometric analysis of whole fresh peripheral blood (PB) samples was performed on a FACSCalibur equiped with the four-color option (Becton Dickinson, CA, USA) as previously described.[17] In particular, the presence of the supposed endothelial and progenitor cells was evaluated in the gate of both $CD31^+CD45^-$ and $CD34^+CD45^-$ cells and on the basis of their further positivity for other endothelial markers such as CD133, CD146, CD105. Platelets or non-specifically stained events, present in the gate of $CD31^+CD45^-$ and identified as $CD61^+CD45^-$ cells, were excluded from the analysis. Negative controls with isotype matched non-relevant monoclonal antibodies (mouse IgG1, IgG2a, IgM) were tested in all experiments. The presence of hematopoietic circulating progenitor cells, was evaluated in the gate of both $CD31^+CD45^+$ and $CD34^+CD45^+$ cells and on the basis of their further positivity for CD184, CD117, CD33, CD4,CD11c antigens.

Table 1. Summary of the clinical characteristic of SSc patients (pts). Patients were untreated at the time of the study.

	Age	*Disease duration (months)*	*Autoantibodies (Anti-topoisomerasi I I/ ACA/ nucleolar/others[a])*	*[b]ILD*	*[c]mRSS (0-51)*
dSSC (12 pts)	48.3 yrs (range 23-60)	38.6 (range 5-60)	6/0/3/3	6	18
lSSC (28 pts)	63.8 yrs (range 37-74)	98 (range 8-125)	1/20/3/4	5	7
Whole group (40 pts)	56 yrs	68.3	7/20/6/7	11	12.5

Some patients were treated with low-doses of steroid until three days before the study. SdSSC: patients affected by a diffuse form of SSc; lSSc: patients affected by a limited form of SSc. [a]others= anti-nuclear antibodies (ANA), with different pattern and anti-extractable nuclear antigen antibodies (ENA); ACA= anti-centromere autoantibodies; [b]ILD= interstitial lung disease, [c]mRSS= modified Rodnan total skin score (Clements P et al. J Rheumatol 1995; 22:1281-85). We considered a Warrick total score of ≥7 as the minimum level for HRCT (lung high resolution computed tomography) abnormalities to be indicative of significant interstitial lung disease. lSSc resulted older than dSSc and healthy subjects (43±7).

Cell culture and adherent clonogenic assay

The PB samples from SSc patients were collected for circulating progenitor cell isolation and the cell culture was performed in four different liquid media.[18] The detection of possible endothelial colonies, were monitored as previously described.[4,19,20] The same PBMNC were also seeded into semisolid medium (Methocult H4434, Stem Cell Technologies Inc., Vancouver, Canada) specific for the hematopoietic long-term culture colony forming unit assay and the clonogenic output of hematopoietic progenitors was evaluated by scoring *in vitro* colonies at day 14.

Immunohistochemical analysis

Adherent cell colonies from SSc patients were studied *in situ* performing: tartrate-resistant acid phosphatase (TRAP), non specific a-naphthyl acetate esterase (NSE) and inhibited a-naphthyl acetate esterase (all from Sigma Aldrich), as recommended by the International Committee for Standardization in Haematology (ICSH) manufacturer's guidelines.[21]

Cytokines assay

The presence and the serum levels of angiogenic cytokines (VEGF, HGF, bFGF, PDGF-BB) were analyzed using a Searchlight human angiogenesis array 2-multiplex assay (Tema Ricerca, Bologna Italy). Stromal derived factor -1 (SDF-1) serum levels were also measured using a single ELISA kit (R&D Systems Inc). Data were calculated using a standard curve generated with specific standards, according to manufacturer's recommendations.

Statistical analysis

The number of freshly detected HCPCs and the number of *in vitro* adherent colonies was compared with the different SSc subsets and clinical features using non-parametric statistics (Wilcoxon test). Data were computed with the Systat™ for Window™. Correlation between the different hematopoietic and non-hematopoietic colonies with clinical and laboratory findings was evaluated using Pearson's test.

Results and Discussion

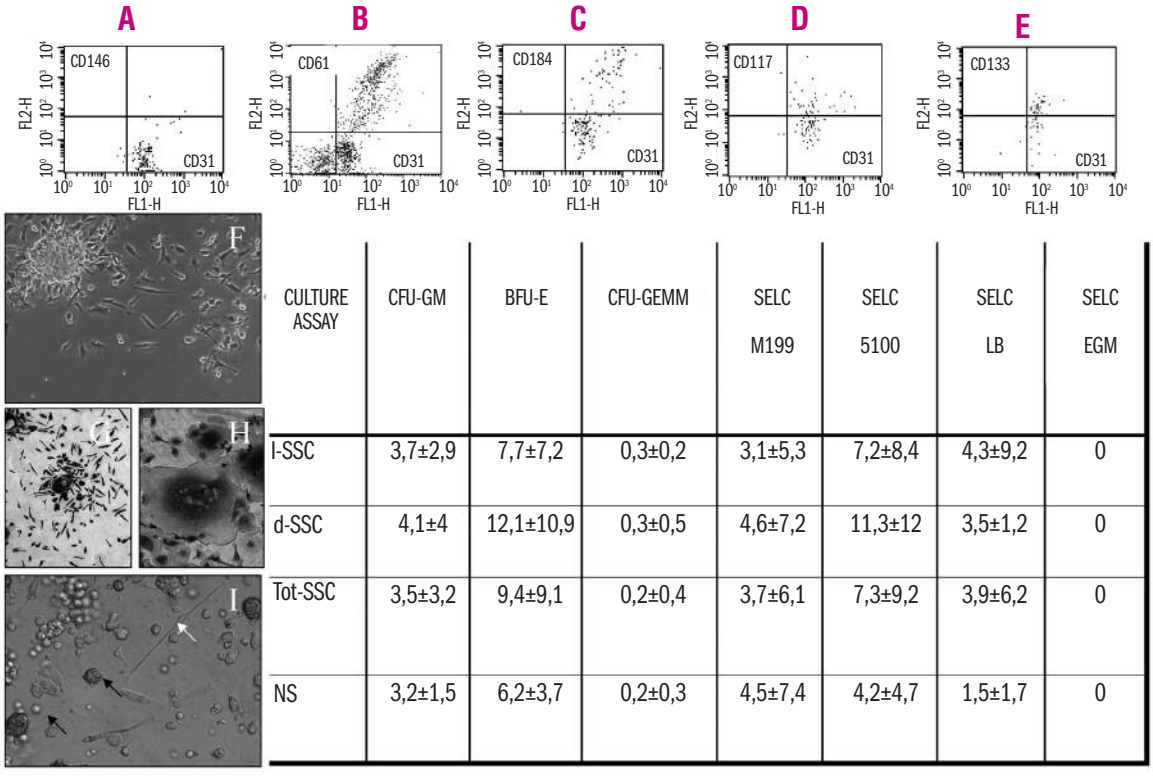
There is still controversy regarding the role of circulat-

ing endothelial and progenitor cells (CECs/CEPs) in the pathogenesis of the systemic sclerosis (SSc). The results are sometimes conflicting due to a phenotypic overlap between hematopoietic and endothelial progenitor cells[13,14] and to the lack of a standardized analytical methodological approach.

In this study, whole fresh PB samples were analyzed by a sequential Boolean gating strategy based on a 4-color cytometric protocol as previously described.[17] The immunophenotypic results showed the lack of in particular CD146$^+$ and CD133$^+$ endothelial, respectively, mature and progenitor CD31$^+$CD45$^-$ endothelial cells either in patients or in normal controls (Figure 1 A). However, in agreement with Strijbos *et al.*[22] a small population of CD31$^+$CD45$^-$ circulating *cells* expressing some endothelial associated markers (SSc patients, whole group: 0.5%±0.4 SD; 0.6%±0.3 SD in l-SSc *vs*. 0.4%±0.4 SD in d-SSc) was observed and excluded from the analysis on the basis of their positivity for platelet associated CD61 marker, high SSC scatter and negativity for CD34 and CD133 thus indicating their non-endothelial derivation (Figure 1 B).

To further investigate the presence of endothelial cells, the immunophenotypic results were combined to different progenitor culture assays [4,19,20] that we routinely use for the growth of CECs and CEPs in patients with hematologic disorders.[19] In this study, progenitor and mature endothelial cells were not detected in any of the samples examined. Furthermore, we did not observe *in vitro* differentiation of circulating progenitor cells towards the endothelial lineage in any patients even in the presence of different angiogenic cytokines for extended long-term cultures (Figure 1, Table of culture assay).

These data, supporting the notion that neither mature or endothelial progenitor cells are detectable in the blood of SSc, are in agreement with the hypothesis of a possible defective vasculogenesis, as previously suggested by other authors.[11] Nevertheless, at day 5-7 small adherent clusters of (10-100 cells) elongated and sprouting cells with a characteristic spindle-shaped endothelial-like (SELC) morphology appeared in liquid culture for long-term hematopoietic cells (Figure 1 F). SELC grow in a granulo-monocytic manner. The cytochemical



CULTURE ASSAY	CFU-GM	BFU-E	CFU-GEMM	SELC M199	SELC 5100	SELC LB	SELC EGM
l-SSC	3,7±2,9	7,7±7,2	0,3±0,2	3,1±5,3	7,2±8,4	4,3±9,2	0
d-SSC	4,1±4	12,1±10,9	0,3±0,5	4,6±7,2	11,3±12	3,5±1,2	0
Tot-SSC	3,5±3,2	9,4±9,1	0,2±0,4	3,7±6,1	7,3±9,2	3,9±6,2	0
NS	3,2±1,5	6,2±3,7	0,2±0,3	4,5±7,4	4,2±4,7	1,5±1,7	0

Figure 1. Whole fresh PB samples were analyzed by a sequential gating strategy based on a 4-color cytometric protocol. (A) Circulating progenitor cells are gated on viable CD34$^+$CD45$^-$ and CD31$^+$CD45$^-$ cell subsets; no supposed endothelial cells were observed in the gate of CD34$^+$CD45$^-$. (B) Platelets or non-specifically stained events, present in the gate of CD31$^+$CD45$^-$ and identified as CD61$^+$CD45$^-$ cells, were excluded from the analysis. (C-D-E) The presence of the hematopoietic circulating progenitor cells was assessed in parallel and analyzed in the gate of CD34$^+$CD45$^+$ for their positivity also for other stem cell hematopoietic and endothelial/monocytic markers (CD184, CD117, CD133). (F) An example of SELC colony, cultured in liquid long-term medium is shown. SELC colonies appeared as spindle/elongated shaped cells, growing in a granulo-monocytic manner, positive for specific and non-specific α-naphthyl acetate esterase (NSE) at day 7 (G). With increasing culture age, SELC tend to fuse by giving rise to a multinucleated giant cells that resulted positive for the specific tartrate-resistance reactivity (TRAP) reaction (H), as expected for osteoclasts. (I) The presence of elongated endothelial-like cells (SELC) are also visible in semisolid medium during short-term clonogenic assay for hematopoietic progenitors (white arrow). Small BFU-E colonies are also visible (black arrows). In the table, the clonogenic output of PB mononuclear cells either in liquid (M199, M5100, LB, EBM) or in semisolid medium (for the growth of colony forming units granulocytes, monocytes and erythroblasts: CFU-GM,CFU-GEMM,BFU-E) is reported and expressed as average ± standard deviation of the number of colonies scored.

reaction for α-naphthyl acetate esterase (NSE) resulted positive on SELC (Figure 1G), but its inhibition with NaF abrogated this positivity, as normally expected for monocytes, thus confirming the monocytic nature of these cells.[13,14] After 30 days of culture, SELCs tended to fuse, giving rise to multinucleated giant cells that resulted positive for the tartrate-resistant acid phosphatase (TRAP) reaction (Figure 1H), as expected for osteoclasts. Although cultured in different liquid media, SELC did not display either *in vitro* adherent expansion capacity or endothelial differentiation potential.

Short-term hematopoietic clonogenic assays revealed a statistically significant increase of circulating erythroid hematopoietic clonogenic potential (Figure 1 I), assessed as a number of cultured (burst forming unit erythroblasts) BFU-E/10^5 PBMNC in the whole SSc patients group (Figure 1 I) (frequency: 35% of limited form, and 46% of the diffuse form) compared with NS (all p values <0.005).

On the other hand, an increased number of circulating hematopoietic progenitor cells (in particular CXCR4 and c-kit-CD117-CD45 positive subsets) co-expressing endothelial and monocytic markers were observed in PB of SSc patients. In particular, a significantly increased level of the different cell subsets gated on $CD34^+CD45^+$ was seen in the PB of SSc compared with that of NS, $CD184^+$ ($CXCR4^+$ HCPCs: 0.047±0.009% SD *vs*. 0.019±0.002% SD in NS, p<0.002); $CD117^+$ (c-kit-HCPCs, also called stem cell factor-R: 0.027±0.008% SD *vs*. 0.015±0.0014% SD in NS, p<0.005), and CD133 (0.018±0.010% SD *vs*. 0.012±0.0014% SD in NS, p>0.003) (Figure 1 C, D, E). Comparable values were found in dSSc and lSSc clinical forms.

Interestingly, in our study, the $CXCR4^+$ hematopoietic progenitor cells subset, implicated in the stem cell homing and mobilization process, was found to be correlated to the presence of cultured SELC, spindle-shaped endothelial-like cell (Pearson test: r=0.79, p<0.005) that resulted positive for endothelial, and myelomonocytic surface markers and that showed a selective optimal outgrowth under hematopoietic culture conditions.

As far as the presence of c-kit -CD117- HCPCs cell subsets is concerned, in SSc patients and, especially in the dSSc subgroups, we further showed that CD117-HCPCs cell subset positively correlated with the number of PB-BFU-E (p<0.005) therefore suggesting a preferential *in vitro* erythroid commitment of this circulating hematopoietic cell subset. These findings are in agreement with evidence that CD31 and CD117 antigens could also be expressed on hematopoietic progenitor cells committed in particular to the erythroid pathway.[23]

High serum levels of angiogenic cytokines (VEGF, PDGF, EGF, IGF) and an increased concentration of SDF-1 chemokine, particularly evident in the dSSc subgroup, were observed (Figure 2), dSSc form differed significantly in the levels of SDF-1 (p<0.003) and to a lesser extent of VEGF (p<0.005) and HGF cytokines (p<0.003) as compared with normal subjects. This, together with an assumed defective vasculogenesis as suggested for SSc, led us to speculate that elevated levels of angiogenic cytokines, could be responsible for promoting the circulating hematopoietic cells erythroid commitment rather than the endothelial one, since in dSSc of the disease the number of BFU-E in PB positively correlated with the levels of SDF-1 (p=0.002) and of circulating CD117-CPCs cell subset (p<0.005). These results are in agreement with the findings in literature supporting the view that these two populations share the same progenitor known as hemoangioblast.[23] However, this finding may also be correlated to a subclinical hypoxiemic status in SSc patients due to pulmonary disease. But no correlation was found between hemoglobin levels and BFU-E count in SSc patients, or between SSc and controls.

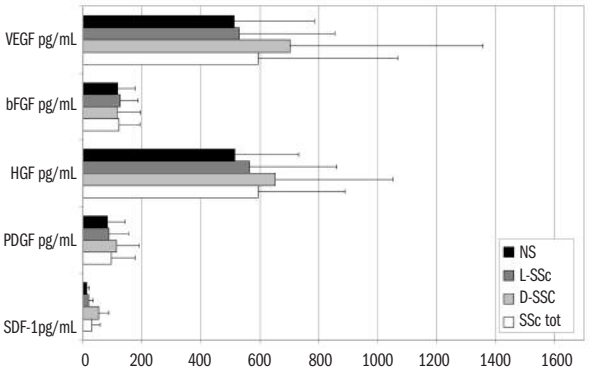


Figure 2. The serum levels of different angiogenic cytokines such as VEGF, PDGF, HGF, bFGF and the chemokine SDF-1, evaluated on the serum of SSc patients is reported. VEGF and HGF displayed the highest serum levels either in normal samples or in SSc patients. SSc patients, in particular d-SSc differed significantly in the levels of SDF-1, and to a lesser extent in the levels of VEGF and HGF cytokines compared with normal subjects.

Clinical data showed that the number of freshly circulating $CXCR4^+$ HCPCs positively correlated with mRSS (p=0.005), pulmonary involvement (p<0.005), and the severity score used to evaluate the interstitial lung disease (p<0.002). A positive correlation among $CXCR4^+$ HCPCs and two factors, implicated in the progenitor cell recruitment such as SDF-1 (p=0.005) and VEGF (p=0.003) were still observed, especially in the diffuse form of SSc.

All these data seem to suggest a possible role for circulating hematopoietic progenitor cells in the fibrotic process of SSc. In particular, high SDF-1/VEGF serum levels could play a role in facilitating the organ homing of $CXCR4^{pos}$-HCPCs and their perivascular positioning and retention. During the course of the disease, if a mechanism for vascular repair is defective, recruited hematopoietic $CXCR\text{-}4^{pos}$ cells that are exposed to angiogenic stimulatory factors could enhance the proliferation of the *in situ* resident endothelial cells or could respond to the microenvironmental conditions, as alternatively polarized monocytes or *fibrocytes*, that we identify as *in vitro* SELC, capable of strong stimulation of the fibrotic process. Our data therefore agree with other findings observed in a murine model of bleomycin-induced pulmonary fibrosis[24] where spindle-shaped fibrocyte-like, resembling our SELC, has been found to play a role in the process of lung fibrosis. In conclusion, the presence of SELC could represent a useful biomarker in fibrotic disease as well as in other pathological conditions.

Authorship and Disclosures

DC and ALM contributed equally to the work; DC performed cell cultures studies, immunohistochemistry and part of flow cytometry analysis; she was also responsible for the supervision of laboratory investigations; FL wrote the paper, and was responsible for the design of the study and data interpretation together with AC and FT. SM and LF performed flow cytometry analysis; MF, RLC and FT supervised data collection and clinical management of patients with multiple sclerosis.

The authors reported no potential conflicts of interest.

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