

Bone Marrow Endothelial Progenitors Are Defective in Systemic Sclerosis

Nicoletta Del Papa,¹ Nadia Quirici,² Davide Soligo,³ Cinzia Scavullo,² Michela Cortiana,³ Chiara Borsotti,² Wanda Maglione,⁴ Denise P. Comina,⁴ Claudio Vitali,⁵ Paolo Fraticelli,⁶ Armando Gabrielli,⁶ Agostino Cortelezzi,³ and Giorgio Lambertenghi-Delilieri³

Objective. Vascular abnormalities represent the main component of the pathobiology of systemic sclerosis (SSc), progressing from structural derangements of the microcirculation with abortive neoangiogenesis to final vessel loss. Since circulating endothelial progenitor cells (EPCs) are important in the vascular repair process, we undertook this study to examine their numbers in the peripheral blood (PB) of SSc patients and to evaluate whether their status is related to impaired quantitative and/or qualitative aspects of the bone marrow (BM) microenvironment.

Methods. Circulating EPCs from 62 SSc patients were evaluated by flow cytometry and characterized as CD45 negative and CD133 positive. BM EPCs, identified as CD133 positive, were isolated from 14 SSc patients and grown to induce endothelial differentiation. In addition, progenitor numbers and functional properties of hematopoietic and stromal compartments were analyzed by various assays.

Results. We found that EPCs were detectable in the PB of patients with SSc, and their number was significantly increased in patients with early-stage disease but not in those with late-stage disease. All of the

examined BM samples contained reduced numbers of EPCs and stromal cells, both of which were functionally impaired. Both endothelial and stromal progenitors expressed vascular endothelial growth factor receptor, indicating that BM is strongly induced to differentiate into the endothelial lineage; furthermore, only BM EPCs from patients with early disease led to endothelial differentiation in vitro.

Conclusion. This study provides the first demonstration that in SSc, there is a complex impairment in the BM microenvironment involving both the endothelial and mesenchymal stem cell compartments and that this impairment might play a role in defective vasculogenesis in scleroderma.

Adult bone marrow (BM) is a rich reservoir of tissue-specific stem and progenitor cells, including a small population of cells known as endothelial progenitor cells (EPCs). Extensive data gathered over the last few years show that BM-derived EPCs are present in the peripheral blood (PB), home to sites of active neovascularization, and differentiate to mature endothelial cells (ECs) in situ, thus contributing to endothelial replacement (1).

A number of cytokines and growth factors and their corresponding surface receptors seem to promote the mobilization of BM EPCs, although their individual roles are still unclear. However, it is known that vascular endothelial growth factor (VEGF), an angiogenic peptide with specific mitogenic activity on EPCs, plays a key role. It binds to its receptor (VEGF receptor 2 [VEGFR-2]) and mediates the further differentiation of BM-derived EPCs to mature adherent endothelial monolayers (2).

The number of circulating EPCs in healthy subjects is relatively low, but this may be influenced by a variety of factors and pathophysiologic conditions. Se-

¹Nicoletta Del Papa, MD: Ospedale G. Pini, University of Milan, and Fondazione Matarelli, Ospedale Fatebenefratelli & Oftalmico, Milan, Italy; ²Nadia Quirici, PhD, Cinzia Scavullo, PhD, Chiara Borsotti, PhD: Fondazione Matarelli, Ospedale Fatebenefratelli & Oftalmico, Milan, Italy; ³Davide Soligo, MD, Michela Cortiana, PhD, Agostino Cortelezzi, MD, Giorgio Lambertenghi-Delilieri, MD: Ospedale Maggiore Policlinico IRCCS, University of Milan, Milan, Italy; ⁴Wanda Maglione, MD, Denise P. Comina, MD: Ospedale G. Pini, University of Milan, Milan, Italy; ⁵Claudio Vitali, MD: Ospedale VillaMarina, Piombino, Italy; ⁶Paolo Fraticelli, MD, Armando Gabrielli, MD: Università Politecnica delle Marche, Ancona, Italy.

Drs. Del Papa and Quirici contributed equally to this work.

Address correspondence and reprint requests to Nicoletta Del Papa, MD, Department of Rheumatology, G. Pini Hospital, Via Pini, 9–20122 Milan, Italy. E-mail: delpapa@gpini.it.

Submitted for publication January 4, 2006; accepted in revised form May 11, 2006.

vere tissue ischemia, vascular injury, or tumor growth are all major stimuli associated with EPC mobilization, recruitment, and homing. In this context, it has been reported that the number of circulating EPCs is much greater in patients experiencing a myocardial infarction (3) or vascular trauma (4). Moreover, the number and function of circulating EPCs may be impaired in some disorders characterized by prolonged chronic endothelial damage, such as chronic coronary artery disease, hypertension, congestive heart disease, and diabetes mellitus (5–9). It has therefore been postulated that impaired vascular repair may contribute to the pathogenesis of these chronic disorders and that a small number of EPCs may also be a risk factor for atherosclerotic plaque instability (9).

It is well known that the microcirculation is the main environment of the pathobiologic processes associated with systemic sclerosis (SSc), which range from considerable initial endothelial derangement with capillary thrombosis, to often abortive reparative neoangiogenesis with abnormal capillary proliferation, and to almost complete loss of vessels in target tissues (10–12). We have recently reported the presence of mature circulating ECs in patients with SSc, which are probably the result of shedding from affected blood vessel walls, and we also found that an increased number of EPCs are detectable in PB, particularly in the early disease stages (13). In contrast, Kuwana et al (14) found that patients with SSc have fewer circulating EPCs than do healthy controls and that the function of the cells is impaired. Nothing is known about the number, function, or maturation of EPCs in BM from patients with SSc or other disorders characterized by chronic vascular injury.

The aim of this study was to further investigate, in a new larger series of patients with SSc, the impaired vasculogenesis by evaluating the number of EPCs in the PB during different phases of the disease. Moreover, in some of these patients, the number and functional phenotypes of endothelial and stromal progenitor cells in the BM were also investigated.

PATIENTS AND METHODS

Patients. This study was approved by the local ethics committee boards, and written informed consent was obtained from all participants. We studied 62 consecutive patients fulfilling the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for SSc (15). Patients were classified as having limited cutaneous SSc (lcSSc) or diffuse cutaneous SSc (dcSSc) on the basis of the criteria of LeRoy et al (16). We excluded patients whose symptoms overlapped those of other connective tissue dis-

eases. Clinical assessment of the study population was performed according to the criteria previously described (17). According to those investigators, disease duration was calculated from the time of the first signs and symptoms related to SSc (Raynaud's phenomenon, puffy hands, sclerodactyly with or without proximal scleroderma, dyspnea, and/or dysphagia). For all SSc patients, disease activity was assessed using the activity indexes of the European Scleroderma Study Group (18,19). According to these criteria, the disease was considered active if the sum of the scores of detected items was ≥ 3 . Disease stages were defined as suggested by Medsger and Steen (20): for early lcSSc, disease duration < 5 years; for intermediate/late lcSSc, disease duration ≥ 5 years; for early dcSSc, disease duration < 3 years; and for intermediate/late dcSSc, disease duration ≥ 3 years.

Thirty-eight of the 62 patients received therapy with intravenous prostanoid. The blood samples for EPC assay were always obtained just before the iloprost infusion and at least 1 month after the previous one. Angiotensin-converting enzyme inhibitors and calcium/channel blockers were discontinued at least 3 weeks before the study. We excluded pregnant women, smokers, persons with diabetes or hypertension, patients with significant hyperlipidemia, and those receiving cholesterol-lowering medications, including statins. None of the patients had received any disease-modifying drugs (i.e., corticosteroids, cyclophosphamide, or methotrexate).

Fifty age- and sex-matched healthy subjects were included as controls in the cytofluorometric study of circulating EPCs. Normal BM samples were collected from 10 healthy donors (median age 38 years, range 22–46 years) for allogeneic BM transplantation.

Antibodies. For the immunocytochemical and flow cytometry analyses, we used fluorescein isothiocyanate (FITC)-labeled lectins from *Ulex europaeus* agglutinin type I (UEA-I; Sigma-Aldrich, Milan, Italy) and the following monoclonal antibodies (mAb): anti-factor VIII/anti-von Willebrand factor (anti-vWF) and anti-CD45 (Dako, Milan, Italy), allophycocyanin (APC)-conjugated anti-kinase insert domain-containing receptor (anti-KDR) (89106; R&D Systems, Minneapolis, MN), anti-Tie-2, anti-E-selectin (anti-CD62E), anti-CD105, anti-vascular cell adhesion molecule 1 (anti-VCAM-1) (anti-CD106), anti-platelet endothelial cell adhesion molecule 1 (anti-CD31), phycoerythrin (PE)-conjugated anti-CD3, and PE-conjugated anti-nerve growth factor receptor (anti-NGFR) (C40-1457) (all from BD Pharmingen, San Jose, CA), FITC-conjugated anti-CD34 (8G12), anti-CD14, FITC-conjugated anti-CD14, APC-conjugated anti-CD19 (SJ25C1), and peridin chlorophyll protein-conjugated anti-CD45 (2D1) (all from Becton Dickinson, Milan, Italy), anti-VE-cadherin (anti-CD144) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-AC133/2 and APC-conjugated anti-AC133 (293C3) (anti-CD133) (Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD146, FITC-conjugated anti-CD146, and PE-conjugated anti-CD146 (anti-P1H12) (Chemicon International, Temecula, CA), FITC-conjugated anti-CD105 (SN6; Serotec, Oxford, UK), anti-low-affinity NGFR (anti-LNGFR) (Me20.4; Upstate Biotechnology, Lake Placid, NY), and FITC-conjugated glycophorin A (Immunotech, Westbrook, ME).

Flow cytometry studies. *Detection of circulating EPCs by flow cytometry.* Circulating endothelial progenitors (defined as CD45-negative and CD34-, VEGFR-2-, and CD133-positive cells) were detected as previously described (13). Five-parameter, 3-color flow cytometry was performed using a FACScan flow cytometer equipped with a 15 mW argon laser, and the data were analyzed using CellQuest software (Becton Dickinson, San Jose, CA).

Flow cytometric analysis of BM cells. A total of 2×10^5 cells were incubated with the selected primary mAb. They were then evaluated using a FACSCalibur instrument and CellQuest software (both from Becton Dickinson).

Detection of soluble VEGF. Plasma VEGF concentrations were assessed by commercial enzyme-linked immunosorbent assays (R&D Systems). The concentrations were then calculated using a standard curve generated by specific standards in accordance with the manufacturer's recommendations.

Isolation of BM low-density mononuclear cells (MNCs). After obtaining informed consent from subjects, an average of 30 ml of BM from 14 SSc patients and 10 healthy controls was collected in heparinized tubes and layered on a Ficoll-Paque gradient (specific gravity 1.077 gm/ml; Nycomed Pharma, Oslo, Norway). The low-density MNCs were resuspended in Iscove's modified Dulbecco's medium (IMDM; BioWhittaker, Caravaggio, Italy) supplemented with 10% fetal bovine serum (FBS; Cambrex Bioscience, Caravaggio, Italy).

BM CD133-positive cell separation. The hematopoietic stem cells and progenitor cells were isolated by positively selecting CD133-expressing cells. Briefly, the BM low-density MNCs were incubated for 30 minutes with the AC133/1 mAb, directly labeled with microbeads, and placed on a column in the midiMACS cell separator (Miltenyi Biotec). The labeled cells were separated using a high-gradient magnetic field and eluted from the column after their removal from the magnet. The cells were counted and assessed for viability by trypan blue dye exclusion, and their purity was determined using a FACSCalibur flow cytometer.

Cultures of CD133-positive BM cells. A total of 3×10^5 CD133-positive enriched cells were plated on fibronectin-coated T-25 tissue culture flasks (Boehringer Mannheim, Milan, Italy), grown in M199 medium (Sigma-Aldrich) supplemented with 10% FBS (Cambrex Bioscience), 50 ng/ml VEGF, 1 ng/ml basic fibroblast growth factor (bFGF), and 2 ng/ml insulin-like growth factor 1 (IGF-1) (all from Peprotech, London, UK), and cultured to confluence at 37°C in a fully humidified atmosphere containing 5% CO₂.

Purification and expansion of UEA-I ECs. The details of this procedure have been previously described (21). Briefly, an average of $2-4 \times 10^6$ cells were incubated with FITC-labeled lectins from UEA-I and then with anti-FITC microbeads (MACS; Miltenyi Biotec) and separated by immunomagnetic sorting. Purified cells were cultured in endothelial medium for 3-4 weeks before characterization.

EC activation. Differentiated ECs were activated by adding 10 ng/ml interleukin-1 α (IL-1 α ; PeproTech) to the cultures for 4 and 24 hours. The expression of CD62E, CD105, CD106, vWF, Tie-2, and KDR was evaluated by immunocytochemistry using the alkaline phosphatase-anti-alkaline phosphatase (APAAP) technique, as previously described (22).

Positive selection of anti-NGFR antibody-positive mesenchymal stem cells (MSCs). The details of this procedure have been previously described (23). Briefly, the low-density MNCs were incubated with the Me20.4 mAb labeling the LNGFR receptor (or p75^{NGFR}), incubated with anti-IgG1 immunomagnetic beads, and placed on a midiMACS column. The positive fraction underwent a second separation step. The cells were then counted and assessed for viability, and their purity was determined by flow cytometry using a PE-conjugated anti-human NGFR antibody (clone C40-1457) which reacts with the 75-kd (or low-affinity) subunit of the nerve growth factor receptor.

Colony-forming units-fibroblastic (CFUs-F). The details of this procedure have been previously described (24). Briefly, 1×10^6 low-density MNCs and 1×10^5 NGFR-positive cells from SSc and normal BM were incubated in IMDM and 20% FBS. After 9 days of culture, the flasks were fixed with methanol and stained with crystal violet. The fibroblast colonies were counted using an inverted microscope at 25 \times magnification. Cell clusters consisting of ≥ 50 fibroblasts were scored as CFU-F colonies.

Functional assays. *Fibroblastic differentiation of MSCs.* In order to induce fibroblastic differentiation, 5×10^4 LNGFR-positive cells were incubated in IMDM supplemented with 20% FBS and 10 ng/ml bFGF (PeproTech) in T-25 flasks and processed as previously described (23). Total expansion ability was evaluated as the fold increase (i.e., the final absolute number of cells obtained when all of the starting cells had expanded to senescence, divided by the number of starting cells).

Adipogenic, osteogenic, and endothelial differentiation of MSCs. To induce adipocyte differentiation, 5×10^4 LNGFR-positive cells were cultured and characterized as previously described (25). To induce osteogenic differentiation, 5×10^4 LNGFR-positive cells were cultured and characterized as previously described (26,27). To induce endothelial differentiation, 1×10^5 NGFR-positive enriched cells were plated on fibronectin-coated T-25 tissue culture flasks in the presence of specific endothelial medium and then purified with lectins from UEA-I, as described above.

Long-term BM cultures. Long-term cultures of BM from the SSc patients and normal controls were established according to the method of Chang et al (28). BM MNCs (2×10^7) were plated in 10 ml of MyeloCult medium (StemCell Technologies, Vancouver, British Columbia, Canada). The cultures underwent weekly demipopulation and exchange of half of the supernatant medium for an equal volume of fresh medium. The cells in the harvested media were counted and assayed for progenitor cells by in vitro colony formation. Stroma formation was evaluated as the percentage of the flask surface covered by stromal cells, with confluence considered to represent 100%.

Progenitor assays. The colony-forming unit-granulocyte-macrophage (CFU-GM) assays were carried out by plating 5×10^4 cells in a methylcellulose culture medium (MethoCult GF H4434; StemCell Technologies). Triplicate dishes were incubated at 37°C in a fully humidified atmosphere containing 5% CO₂. After 14 days of culture, aggregates of ≥ 40 cells were scored as colonies and counted.

Statistical analysis. Circulating EPC levels in SSc patients and healthy controls were compared using the Mann-

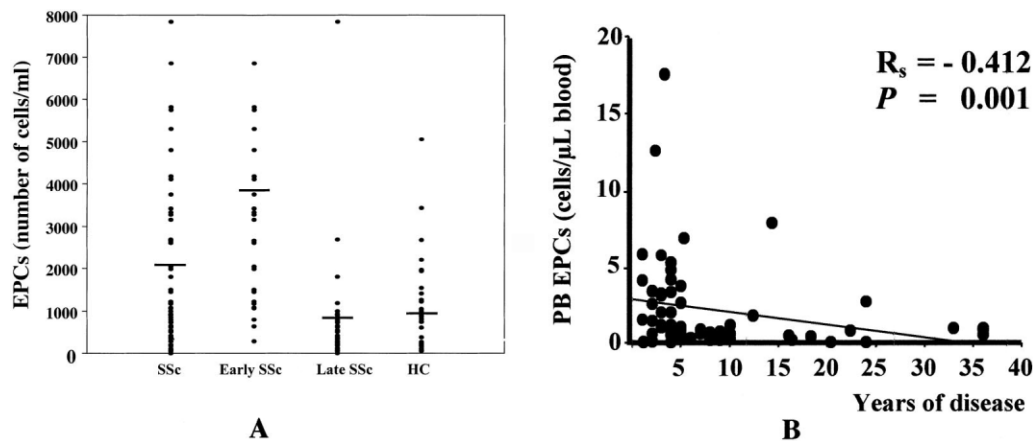


Figure 1. Flow cytometry evaluation of circulating endothelial progenitor cells (EPCs) in the peripheral blood (PB) of patients with systemic sclerosis (SSc). **A**, Levels of EPCs in SSc patients and healthy controls (HC). $P = 0.0042$ for all SSc patients versus healthy controls; $P < 0.0001$ for patients with early SSc versus patients with late SSc. **B**, Correlation of circulating EPC levels with disease duration.

Whitney U test, and correlations between the number of circulating EPCs and disease duration were assessed using Spearman's rank correlation test. P values less than 0.05 were considered significant. BM cell surface marker levels were compared using Student's 2-tailed t -test for paired data.

RESULTS

Demographic and clinical characteristics of the SSc patients. The study included 59 women and 3 men with a median age of 55 years (range 21–72 years); 27 patients were classified as having lcSSc and 35 as having dcSSc. The median disease duration was 5.0 years (range 1–36 years). On the basis of Medsger and Steen's criteria (20), 13 of the 27 patients with lcSSc (48.1%) and 14 of the 35 with dcSSc (40.0%) had early disease. The presence of Raynaud's phenomenon was recorded in all of the patients. Cutaneous ulcers were found in 23 of 62 patients (37.1%). With respect to pulmonary involvement, 11 of the 62 SSc patients had primary pulmonary hypertension and 18 had pulmonary fibrosis with or without pulmonary hypertension. The evaluation of disease activity according to the European Scleroderma Study Group criteria indicated that 21 of the 62 patients had an active disease.

Quantitative studies of PB EPCs. Following the method initially described by Rafii (29), we identified EC progenitors in PB by the surface expression of CD34, VEGFR-2 (KDR), and CD133, a stem cell marker that is not expressed by mature ECs. The mean number of circulating EPCs in the SSc patients (2,108 cells/ml, 95% confidence interval [95% CI] 1,336–2,880) was signifi-

cantly higher than that in the healthy controls (1,027 cells/ml, 95% CI 615–1,439) ($P = 0.0042$) (Figure 1A). Interestingly, when the patients with SSc were stratified on the basis of disease duration, the number of circulating EPCs was significantly increased in those with recent-onset disease (<5 years for lcSSc and <3 years for dcSSc) compared with those with chronic disease ($P < 0.0001$); in addition, the patients with chronic disease (either lcSSc or dcSSc) had lower EPC counts than the healthy controls, but this difference was not statistically significant (862 cells/ml versus 1,027 cells/ml) (Figure 1A). There was a close negative correlation between the number of circulating EPCs and disease duration ($r_s = -0.412$, $P = 0.0013$) (Figure 1B). No significant correlation with clinical or pathologic parameters, namely, digital ulcers, skin and visceral involvement, and disease activity score, was found. No significant correlation between EPC counts and concomitant vascular therapies was noted.

Soluble VEGF analysis. VEGF is known to be a major angiogenic peptide involved in a number of the steps of angiogenesis, including the proliferation and recruitment of EPCs from BM (1,2). Mean plasma VEGF levels were higher in the SSc patients (601 pg/ml, range 81–1,348 pg/ml) than in the healthy controls (101.3 pg/ml, range undetectable–191 pg/ml) ($P < 0.001$). Interestingly, mean VEGF levels tended to be higher in patients with advanced disease than in those with recent-onset disease (768.2 pg/ml versus 432.8 pg/ml) (Figure 2), although no significant differences were found in the 2 different subsets of disease.

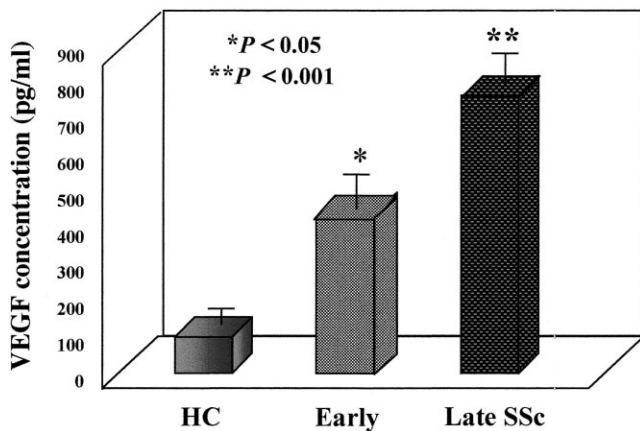


Figure 2. Plasma levels of vascular endothelial growth factor (VEGF) in patients with SSc. Plasma concentrations of VEGF were increased in patients with SSc compared with those in healthy controls and tended to be further increased in advanced disease as compared with recent-onset disease. Values are the mean and SD. *P* values are versus healthy controls. See Figure 1 for other definitions.

No correlation was found between the number of circulating EPCs and VEGF levels.

Phenotype analysis of BM low-density MNCs.

We studied BM samples from 14 SSc patients (3 with early lcSSc, 4 with late lcSSc, 4 with early dcSSc, and 3 with late dcSSc). The mean circulating EPC level was 2,949 cells/ml, with no significant differences between patients with dcSSc and those with lcSSc. When considered according to disease duration, SSc patients with recent-onset disease showed an increased number of circulating EPCs in comparison with patients with chronic disease ($P = 0.003$), as observed in the larger patient group.

In order to determine the phenotype of BM cells in SSc, samples from 14 patients were analyzed by flow cytometry. Compared with normal controls, SSc patients showed a significant decrease in CD133-positive cells (mean \pm SD $0.36 \pm 0.44\%$ versus $1.23 \pm 0.78\%$; $P = 0.01$) and a marked increase in P1H12, the surface marker of mature ECs (mean \pm SD $1.11 \pm 0.6\%$ versus $0.51 \pm 0.07\%$; $P = 0.007$), and in the transforming growth factor β receptor CD105 (mean \pm SD $9.89 \pm 5.7\%$ versus $4.45 \pm 2.7\%$; $P = 0.01$). NGFR, the p75 antigen initially identified in the central nervous system but now known to be expressed in early MSCs, was also down-regulated in SSc patients (mean \pm SD $0.73 \pm 0.47\%$ versus $1.4 \pm 0.64\%$; $P = 0.01$). The levels of all other lineage markers were similar in patients and controls.

The mean \pm SD expression of SSc and normal

BM low-density MNC markers is summarized in Table 1. In none of the patients did we find any alteration of the hemocytometric profile, such as anemia, leukopenia, or thrombocytopenia, nor did we find other clinical or hematologic signs indicating the possibility of myelofibrosis, which could justify "per se" the BM abnormalities found.

BM cultures. BM aspirates were studied in order to evaluate the characteristics of both the hematopoietic and stromal cell compartments. The cells were cultured in long-term BM cultures, an in vitro situation that more closely mimics the BM in vivo. The production of hematopoietic cells in the supernatant (evaluated by weekly demipopulation) was always lower in the examined BM samples from the SSc patients than in those from the normal controls; stromal growth was also deficient, and the cells never reached confluence. With regard to the hematopoietic progenitor evaluations, the median number of CFUs-GM in SSc BM peaked during the first week, followed by a decrease in the third week, whereas the median number in the control long-term BM cultures initially diminished and reached higher values between the third and fourth weeks (Figure 3).

EPC separation and EC differentiation of BM cells. Recovery of CD133-positive cells after immunomagnetic separation was always lower in SSc patients than in normal controls (mean \pm SD $0.12 \pm 0.1\%$ versus $0.35 \pm 0.18\%$; $P = 0.006$), with a purity of $88 \pm 4.8\%$ as determined by flow cytometry. In order to induce endothelial differentiation of BM cells, we used a previously reported technique (21) based on CD133-positive cell immunoseparation and culture in the presence of

Table 1. Low-density MNC fluorescence-activated cell sorter analysis*

	Normal BM	SSc BM	<i>P</i>
CD45	87.1 \pm 9.3	92.2 \pm 5.8	-
CD133	1.23 \pm 0.78	0.36 \pm 0.44	0.01
CD134	2.98 \pm 1.53	1.96 \pm 0.5	-
Glycophorin A	9.86 \pm 6.4	5.48 \pm 5	-
CD105	4.45 \pm 2.7	9.89 \pm 5.7	0.01
CD14	10.2 \pm 4.35	6.79 \pm 3.4	-
CD3	18.9 \pm 7.7	21.1 \pm 9.8	-
CD19	4.02 \pm 2.65	4.72 \pm 3	-
CD117	3.44 \pm 1.2	3.55 \pm 3.6	-
NGFR	1.4 \pm 0.64	0.73 \pm 0.47	0.01
P1H12	0.51 \pm 0.07	1.11 \pm 0.6	0.007
KDR	0.32 \pm 0.23	0.41 \pm 0.27	-

* Values are the mean \pm SD percentage of labeled cells in the mononuclear cell (MNC) fractions of 15 normal bone marrow (BM) samples and 14 BM samples from patients with systemic sclerosis (SSc). Only significant *P* values are shown. NGFR = nerve growth factor receptor; KDR = kinase insert domain-containing receptor.

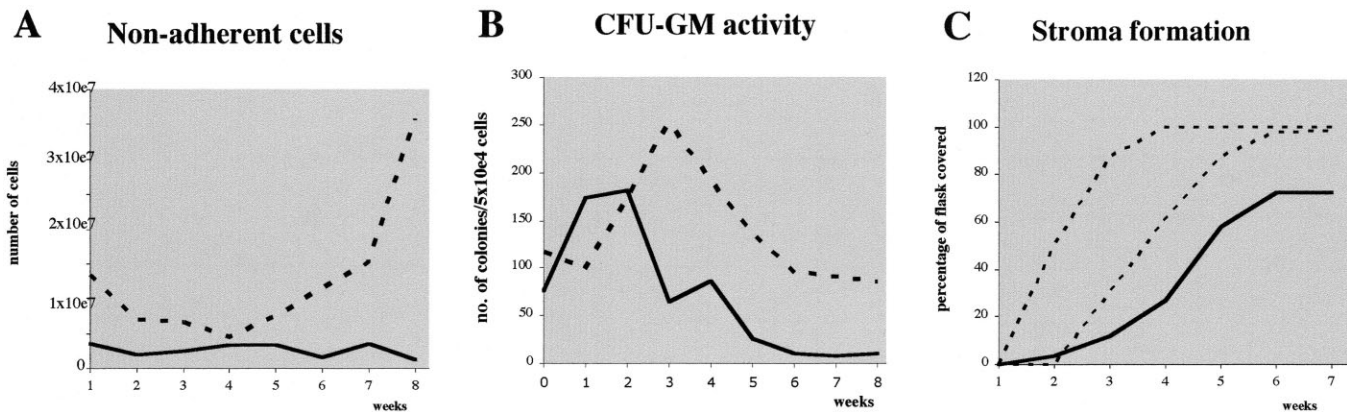


Figure 3. Evaluation of the ability of bone marrow (BM) from patients with systemic sclerosis (SSc) to produce long-term cultures in vitro. Low-density mononuclear cells from SSc patients are deficient in the ability to form long-term BM cultures. **A**, Median number of recovered nonadherent cells from weekly demipopulation in the supernatant of long-term BM cultures from SSc patients (solid line) and normal controls (broken line). **B**, Median number of colony-forming units–granulocyte–macrophage (CFUs-GM), at 1-week intervals, from the nonadherent fraction of long-term BM cultures from SSc patients (solid line) and normal controls (broken line). **C**, Median percentage of stroma formation, at 1-week intervals, in SSc patients (solid line). The area between the broken lines represents the range of stroma formation in long-term BM cultures from normal controls.

VEGF, and a second step of immunomagnetic purification using FITC-labeled lectins from UEA-I and anti-FITC microbeads. This method makes it possible to obtain a pure EC population with a high proliferative potential (i.e., a mean expansion capacity that is $2,228 \pm 375$ -fold that of UEA-I-purified cells).

The separated CD133-positive cells were grown

on fibronectin-coated flasks in the presence of VEGF, bFGF, and IGF-1. After 21 days of culture, the cells had formed some colonies in only 4 of 14 cases. In 2 of these cases (both involving cells from patients with late lcSSc), the colonies were rare and small and did not expand, and the cells rapidly showed aging and signs of stress (Figure 4); in the other 2 cases (both involving cells from

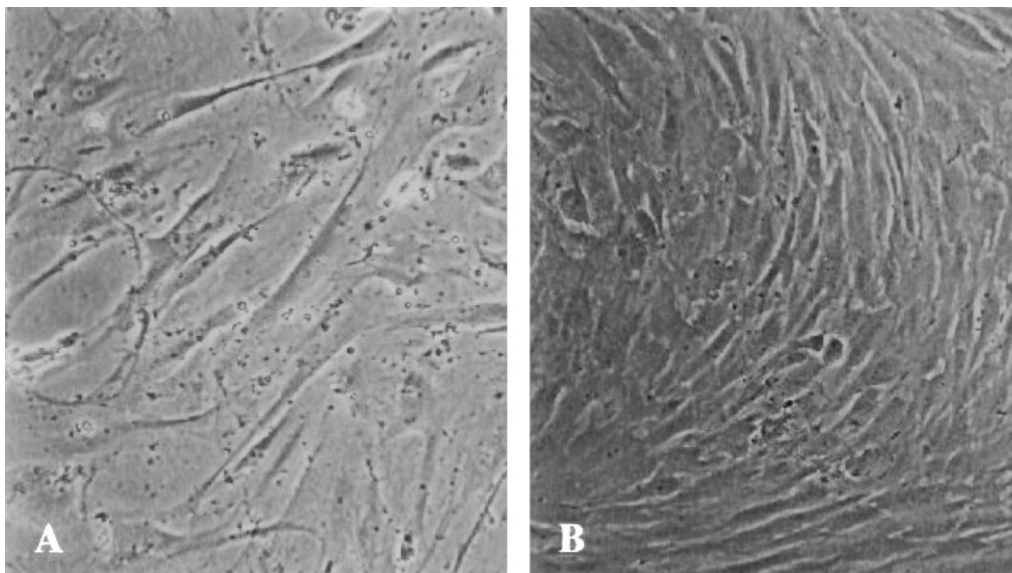


Figure 4. Phase-contrast micrographs of endothelial cell cultures. CD133-positive immunoselected cells after 3 weeks of culture grew in only 4 of 14 systemic sclerosis (SSc) bone marrow (BM) samples, but the colonies from late SSc BM samples were rare and small and did not expand, and the cells rapidly showed aging and signs of stress (**A**). In normal control BM samples (**B**), the cells reached confluence and had elongated spindle-shaped morphology with focal areas of cobblestone-like cells. (Original magnification $\times 100$.)

patients with early SSc), there were numerous colonies of cells characterized by a round central body with some cytoplasmic projections which, when they reached confluence, consisted of a monolayer of spindle-shaped cells with some areas of cell aggregates with the typical "cobblestone" morphology of ECs. However, their purification with lectins from UEA-I and immunomagnetic sorting was ineffective; the obtained cells (5.5×10^3 UEA-I-positive cells) were plated on fibronectin-coated flasks and cultured in the presence of VEGF, but did not expand and died within a few days. Their final total expansion capacity was therefore 350-fold, much lower than that of the cells from normal controls.

BM stromal cells. In order to evaluate the number of stromal cell precursors in BM, we used a CFU-F assay that identifies a population of rapidly-adherent clonogenic cells with a fibroblastic morphology, which are capable of extended proliferation and multilineage differentiation in vitro (23). In the normal control BM samples, the number of CFUs-F varied but was significantly higher than that observed in the 14 SSc BM samples (36.3 ± 19.4 colonies/ 1×10^6 seeded cells versus 9.8 ± 13 colonies/ 1×10^6 seeded cells; $P = 0.0019$).

In BM, the NGFR antigen defines a subset of early MSCs with a high proliferative potential, a high degree of clonogenic efficiency, and the ability to differentiate into multiple mesodermal tissues. In order to obtain a population of homogeneous and multipotent MSCs, we positively selected low-affinity anti-NGFR antibodies as previously reported (23).

After immunomagnetic separation, the mean \pm SD recovery of NGFR-positive cells in 8 SSc BM samples was $0.64 \pm 0.3\%$ compared with $1.2 \pm 0.6\%$ in normal BM samples ($P = 0.029$), with a purity of $88 \pm 4.5\%$ as determined by flow cytometry. Phenotype analysis showed no significant differences in the examined surface markers except for the overexpression of KDR in SSc BM samples ($23.1 \pm 7.8\%$ versus $4.6 \pm 1.7\%$; $P = 0.002$). The clonogenic efficiency of the NGFR-positive cells was always less in the SSc BM samples than in the normal control BM samples (7 ± 12.8 colonies/ 1×10^5 seeded cells versus 69 ± 61 colonies/ 1×10^5 seeded cells; $P = 0.01$).

NGFR-positive cells are multipotent and capable of differentiating into mesenchymal tissue lineages, including stroma, adipose, and bone. In order to evaluate their ability to differentiate to fibroblasts, they were grown in the presence of bFGF, but formed a confluent layer with a fibroblast morphology in only 3 of 8 cases (all involving cells from patients with early dcSSc).

Table 2. Phenotype characterization of the endothelial differentiation of CD133-positive and NGFR-positive precursors from BM samples from patients with early SSc and normal controls*

	Differentiated SSc BM ECs	Differentiated normal BM ECs
vWF	89.7 ± 9.7	99.3 ± 0.7
CD105	98 ± 1	100
VCAM-1	93.3 ± 6.7	0
P1H12	86.7 ± 2.5	100
VE-cadherin	89.3 ± 3.5	99.2 ± 0.6
CD31	93 ± 5.6	99.6 ± 0.5
CD62E	89 ± 3.6	0
CD14	<1	0
CD45	<1	0

* Values are the mean \pm SD percentage of labeled cells and are the results of 6 experiments. The reactivity of differentiated cells was evaluated by alkaline phosphatase-anti-alkaline phosphatase staining. The percentage of positive cells was calculated by counting a minimum of 400 cells at $400\times$ magnification. All markers analyzed were intensely expressed. ECs = endothelial cells; vWF = von Willebrand factor; VCAM-1 = vascular cell adhesion molecule 1 (see Table 1 for other definitions).

Compared with NGFR-positive cells from normal controls, the SSc NGFR-positive cells showed signs of rapid aging and stress and had a greatly reduced proliferative capacity (mean \pm SD fold expansion rate $7.3 \times 10^3 \pm 1.3 \times 10^4$ versus $6.2 \times 10^8 \pm 8.2 \times 10^8$). When grown in adipogenic or osteogenic media, the SSc NGFR-positive cells never differentiated.

We have previously demonstrated that normal BM NGFR-positive cells are capable of differentiating to the endothelial phenotype, with total expansion capacities similar to those of CD133-positive differentiated ECs (30). When grown in the presence of VEGF, the SSc NGFR-positive fractions gave rise to endothelial colonies; once again, however, they were unable to expand after UEA-I selection, with a total expansion ability of 26.3 ± 15.7 -fold.

Immunocytochemical analysis of ECs. In vitro-differentiated ECs from early SSc CD133-positive and NGFR-positive precursors were then analyzed for the presence of endothelial markers on APAAP-immunostained cytospin preparations. Like normal mature ECs, almost all of the cells intensely expressed CD105 endoglin, P1H12, vWF, VE-cadherin (CD144), and CD31 (Table 2).

In order to detect the receptors and adhesion molecules typically modulated by ECs stimulated by inflammatory cytokines, the cells were incubated with IL-1 α and analyzed immunocytochemically; vWF, CD62E, and VCAM-1 were up-regulated upon stimulation. Interestingly, in SSc patients, VCAM-1 (CD106),

CD105 endoglin, and CD62E were intensely expressed even before activation.

DISCUSSION

Many studies have shown that, despite severe tissue ischemia, new microvessel formation is defective in SSc patients and is unable to replace the damaged capillaries (12). Since the results of a number of experimental studies indicate that circulating EPCs are important to the process of vascular repair (2), we examined their role in scleroderma vasculogenesis by investigating the number of EPCs in the PB of patients with both early- and late-phase SSc, as well as their number and functional aspects in BM.

The results of the study confirm that EPCs are detectable in the PB of SSc patients, that their number is significantly increased in patients with early-phase disease but not in those with late-phase disease, and that the levels of VEGF (the main cytokine driving the differentiation, proliferation, and mobilization of EPCs) are significantly higher in SSc patients, particularly those with late-phase disease. Regarding the examination of BM aspirated from SSc patients, the main results we obtained indicate the following: 1) regardless of the disease phase, the BM of all SSc patients examined contained much fewer and functionally impaired EPCs; 2) only the BM EPCs from patients with early disease gave rise to some degree of *in vitro* endothelial differentiation; 3) the stromal compartment of SSc BM was severely defective in stem cells, and their function was also impaired; and 4) the surfaces of both endothelial and stromal progenitors expressed the VEGF receptor, thus showing that BM is strongly directed toward differentiating into the endothelial lineage.

These findings confirm in an additional group of patients our preliminary data (13) and only apparently conflict with those reported by Kuwana et al (14), who found a reduced number of EPCs with impaired function in a small series of patients with mainly late-phase SSc; the conflict with the findings of Kuwana et al is superficial, in so far as our patients with advanced SSc also had a limited number of circulating EPCs, thus indicating a probable exhaustion of the precursor endothelial pool, as has been previously suggested in the case of other chronic vascular disorders (2,6). This conclusion is further supported by the increased VEGF levels observed in our patients, as reported in previous studies (31,32). Furthermore, the fact that VEGF levels progressively increased from the early to the late phase of the disease can be interpreted to be the result of an

impaired target cell response that becomes increasingly severe as the disease progresses. Interestingly, Dor et al (33) showed in an experimental animal model that the overexpression of VEGF induced the formation of new vessels in adult organs; however, the prolonged exposure resulted in a chaotic vascular morphology with reduced blood flow in the newly formed vessels. In addition, Distler et al (34) indicated that a chronic and uncontrolled overexpression of VEGF does occur in SSc and might significantly contribute to the chaotic capillary morphology seen in these patients.

The possibility that the target organ of this powerful angiogenic "push" cannot satisfy the continuous and prolonged peripheral demand is confirmed by the quantitative impairment of the endothelial stem cell source in the BM of all of our SSc patients. In addition, only the BM endothelial progenitors from patients with early disease led to endothelial differentiation *in vitro*, thus suggesting that residual EPC recruitment still takes place during the early stages of the disease. Taken together, these findings are consistent with the inverse correlation we found between the number of PB EPCs and disease duration. The fact that we found the same characteristics in all of the SSc BM samples studied (with no differences related to disease duration) suggests that the exhaustion of resident BM endothelial progenitors may be a very early step in the course of the disease.

It has been shown that mesenchymal stem cells and progenitor cells can differentiate to an endothelial phenotype and enhance vascularization (35,36), and we have previously demonstrated that NGFR-positive cells can differentiate *in vitro* to the endothelial phenotype when grown in the presence of VEGF (30). The overexpression of angiogenic receptors shown by SSc mesenchymal progenitors may be an extreme attempt to increase EPC mobilization from BM, a hypothesis supported by the fact that in the presence of VEGF, these multipotent cells preferentially gave rise to endothelial differentiation, thus suggesting the possible existence of a response to increasing endothelial requirements largely involving BM cell populations. Observations that the transplantation of SSc skin biopsy specimens into immunodeficient mice or the chick embryo chorioallantoic membrane generates increased angiogenic responses (37,38) also support the hypothesis that the mechanisms capable of triggering neoangiogenesis can be up-regulated in SSc and that the impaired repair of vascular tissue is probably due to an inadequate response of SSc BM.

An alternative to the hypothesis that prolonged

and continuous EC recruitment may exhaust the BM reservoir of resident EPCs is that disease-related cytotoxic mechanisms negatively influence the half-life and mobilization of BM EPCs. Since EPCs share the phenotypic and functional properties of mature ECs (39), they could be the target of antiendothelial antibodies, which have been shown to induce apoptotic phenomena and an activated proinflammatory phenotype in mature SSc ECs (40,41). The overexpression of surface adhesion molecules by resting and activated differentiated EPCs from SSc BM seems to support this hypothesis. Activation of ECs, as defined by increased expression of adhesion molecules, has been described in the SSc microvasculature. E-selectin and intercellular adhesion molecule 1 (ICAM-1) expression have been found to be up-regulated in the skin of SSc patients (42–46). In addition, increased plasma concentrations of soluble forms of EC surface adhesion molecules, including ICAM-1, VCAM-1, and E-selectin, have been described in several studies (47,48).

Although our findings are related to observations of the *in vitro* differentiation of EPCs into mature ECs, they provide further evidence for the relevance of EC activation (even in SSc BM) and possible grounds for impaired peripheral incorporation of ECs. Together with the modulation of cytokine, chemokine, and growth factor profiles, the additional immunologic abnormalities related to repertoires and activities of B and T cells are thought to contribute to the inability to resolve inflammation and the failure of reparative processes (49).

We can further speculate that the complex impairment of both endothelial and stromal progenitors observed in the BM of our patients may be due to the severe generalized microvascular damage associated with SSc also involving the BM vasculature. We and other investigators (13,50) have previously reported that the presence of circulating mature ECs in SSc probably provides direct evidence of vascular injury leading to EC shedding. We found an increased number of circulating mature ECs in the BM of SSc patients compared with normal controls, and this is consistent with the widespread endothelial damage affecting sclerodermatous tissues.

In addition to all the possible hypotheses raised to explain our findings, we must remember that defects of EC lineages are regarded as the first crucial moment in the pathogenetic cascade in SSc. In this regard, we can look at EPC dysfunction as a consequence, rather than a primary event.

In conclusion, the results of our study do not

allow any definite conclusions concerning the mechanisms accounting for the complex BM scenario in SSc patients, and it is possible that all of our hypotheses are simultaneously true. However that may be, BM cells seem to be crucial in the pathogenesis of SSc and may have a number of future therapeutic applications. BM could be a novel target for drugs capable of enhancing the mobilization and migration of EPCs, and in this context, recent clinical studies have shown that statins can significantly increase the number of circulating EPCs (51,52). Finally, the transplantation of autologous or exogenous BM-derived endothelial progenitors may contribute to restoring sclerodermatous tissue vascularization (at least in early SSc), just as recent pioneering clinical studies have shown that they are effective in accelerating organ vascularization and repair after ischemic events in the limbs, retina, and myocardium (2,53).

To the best of our knowledge, this is the first study of endothelial progenitors in their original sites, and we cannot exclude the possibility that similar BM abnormalities may be found in other chronic vascular diseases. Our findings provide the first demonstration that in SSc patients, there is a complex BM microenvironment impairment involving both the endothelial and MSC compartments and that this impairment may be involved in the defective EPC-dependent neoangiogenesis.

REFERENCES

1. Murasawa S, Asahara T. Endothelial progenitor cells for vasculogenesis. *Physiology (Bethesda)* 2005;20:36–42.
2. Hristov M, Weber C. Endothelial progenitor cells: characterization, pathophysiology, and possible clinical relevance. *J Cell Mol Med* 2004;8:498–508.
3. Shintani S, Murohara T, Ikeda H, Ueno T, Hoenma T, Katoh A, et al. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. *Circulation* 2001;103:2776–9.
4. Gill M, Dias S, Hattori K, Rivera ML, Hicklin D, Witte L, et al. Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells. *Circ Res* 2001;88:167–74.
5. Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res* 2001;89:e1–7.
6. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 2003;348:593–600.
7. Valgimigli M, Rigolin GM, Fucili A, Della Porta M, Soukhomovskaia O, Malagutti P, et al. CD34⁺ and endothelial progenitor cells in patients with various degrees of congestive heart failure. *Circulation* 2004;110:1209–12.
8. Loomans CJ, de Koning EJ, Staal FJ, Rookmaaker MB, Verseyden C, de Boer HC, et al. Endothelial progenitor cell dysfunction:

- a novel concept in the pathogenesis of vascular complications of type 1 diabetes. *Diabetes* 2004;53:195–9.
9. Werner N, Kosiol S, Schiegl T, Ahlers P, Walenta K, Link A, et al. Circulating endothelial progenitor cells and cardiovascular outcomes. *N Engl J Med* 2005;353:999–1007.
 10. Herrick AL. Vascular function in systemic sclerosis. *Curr Opin Rheumatol* 2000;12:527–33.
 11. LeRoy EC. Systemic sclerosis: a vascular perspective. *Rheum Dis Clin North Am* 1996;22:675–94.
 12. Flavahan NA, Flavahan S, Mitra S, Chotani MA. The vasculopathy of Raynaud's phenomenon and scleroderma. *Rheum Dis Clin North Am* 2003;29:275–91.
 13. Del Papa N, Colombo G, Fracchiolla N, Moronetti LM, Ingegnoli F, Maglione W, et al. Circulating endothelial cells as a marker of ongoing vascular disease in systemic sclerosis. *Arthritis Rheum* 2004;50:1296–304.
 14. Kuwana M, Okazaki Y, Yasuoka H, Kawakami Y, Ikeda Y. Defective vasculogenesis in systemic sclerosis. *Lancet* 2004;364:603–10.
 15. Subcommittee for Scleroderma Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Preliminary criteria for the classification of systemic sclerosis (scleroderma). *Arthritis Rheum* 1980;23:581–90.
 16. LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA, et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. *J Rheumatol* 1988;15:202–5.
 17. Ferri C, Valentini G, Cozzi F, Sebastiani M, Michelassi C, La Montagna G, et al. Systemic sclerosis: demographic, clinical, and serologic features and survival in 1,012 Italian patients. *Medicine (Baltimore)* 2002;81:139–53.
 18. Valentini G, Della Rossa A, Bombardieri S, Bencivelli W, Silman AJ, D'Angelo S, et al. European multicentre study to define disease activity criteria for systemic sclerosis. II. Identification of disease activity variables and development of preliminary activity indexes. *Ann Rheum Dis* 2001;60:592–8.
 19. Valentini G, Bencivelli W, Bombardieri S, D'Angelo S, Della Rossa A, Silman AJ, et al. European Scleroderma Study Group to define disease activity criteria for systemic sclerosis. III. Assessment of the construct validity of the preliminary activity criteria. *Ann Rheum Dis* 2003;62:901–3.
 20. Medsger TA Jr, Steen VD. Classification, prognosis. In: Clements PJ, Furst DE, editors. *Systemic sclerosis*. 2nd ed. Philadelphia: Lippincott Williams & Wilkins; 1996. p. 51–79.
 21. Quirici N, Soligo D, Caneva L, Servida F, Bossolasco P, Delilieri GL. Differentiation and expansion of endothelial cells from human bone marrow CD133⁺ cells. *Br J Haematol* 2001;115:186–94.
 22. Cordell JL, Falini B, Erber WN, Ghosh AK, Abdulaziz Z, MacDonald S, et al. Immunoenzymatic labelling of monoclonal antibody using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). *J Histochem Cytochem* 1984;32:219–29.
 23. Quirici N, Soligo D, Bossolasco P, Servida F, Lumini C, Delilieri GL. Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. *Exp Hematol* 2002;30:783–91.
 24. Castro-Malaspina H, Gay RE, Resnick G, Kapoor N, Mayers P, Chiarirei D, et al. Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. *Blood* 1980;56:289–301.
 25. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;284:143–7.
 26. DiGirolamo CM, Stokes D, Colter D, Phinney G, Class R, Prockop DJ. Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. *Br J Haematol* 1999;107:275–81.
 27. Stanford CM, Jacobson PA, Eanes ED, Lembke MA, Midura LJ. Rapid forming apoptotic mineral in an osteoblastic cell line (UMR 106-01 BSP). *J Biol Chem* 1995;270:9420–8.
 28. Chang J, Morgenstern G, Coutinho LH, Scarffe JH, Carr T, Deakin DP, et al. The use of bone marrow cells grown in long-term bone marrow cultures of autologous BMT in acute leukaemia: an update. *Bone Marrow Transplant* 1989;4:5–9.
 29. Rafii S. Circulating endothelial precursors: mystery, reality, and promise. *J Clin Invest* 2000;105:17–9.
 30. Quirici N, Soligo D, Borsotti C, Scavullo C, Zangrossi S, Lambertenghi-Delilieri G. Phenotypic and molecular characterization of bone marrow L-NGFR⁺ mesenchymal stem cells [abstract]. *Blood* 2004;104:2333.
 31. Distler O, Del Rosso A, Giacomelli R, Cipriani P, Conforti ML, Guiducci S, et al. Angiogenic and angiostatic factors in systemic sclerosis: increased levels of vascular endothelial growth factors are a feature of the earliest disease stages and are associated with the absence of fingertip ulcers. *Arthritis Res* 2002;4:R11.
 32. Choj JJ, Min DJ, Cho ML, Min SY, Kim SJ, Lee SS, et al. Elevated vascular endothelial growth factor in systemic sclerosis. *J Rheumatol* 2003;30:1529–33.
 33. Dor Y, Djonov V, Abramovitch R, Itin A, Fishman GI, Carmeliet P, et al. Conditional switching of VEGF provides new insights into adult neovascularization and pro-angiogenic therapy. *EMBO J* 2002;21:1939–47.
 34. Distler O, Distler JH, Schaid A, Acker T, Hirth A, Rethage J, 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.
 35. Davani S, Marandin A, Mersin N, Royer B, Kantelip B, Herve P, et al. Mesenchymal progenitor cells differentiate into an endothelial phenotype, enhance vascular density and improve heart function in a rat cellular cardiomyoplasty model. *Circulation* 2003;108:11253–8.
 36. Gojo S, Gojo N, Takeda Y, Mori T, Abe H, Kyo S, et al. In vivo cardiovascularogenesis by direct injection of isolated adult mesenchymal stem cells. *Exp Cell Res* 2003;288:51–9.
 37. Dong C, Zhu S, Wang T, Yoon W, Li Z, Alvarez RJ, et al. Deficient Smad7 expression: a putative molecular defect in scleroderma. *Proc Natl Acad Sci U S A* 2002;99:3908–13.
 38. Ribatti D, Cantatore FP, Vacca A, D'Amore M, Ria R, Roncali R, et al. Systemic sclerosis stimulates angiogenesis in the chick embryo chorioallantoic membrane. *Clin Rheumatol* 1998;17:115–20.
 39. Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, et al. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. *Blood* 2000;95:952–58.
 40. Sgonc R, Gruschwitz MS, Boeck G, Sepp N, Gruber J, Wick G. Endothelial cell apoptosis in systemic sclerosis is induced by antibody-dependent cell-mediated cytotoxicity via CD95. *Arthritis Rheum* 2000;43:2550–62.
 41. Lunardi C, Bason C, Navone R, Millo E, Damonte G, Corrocher R, et al. Systemic sclerosis immunoglobulin G autoantibodies bind the human cytomegalovirus late protein UL94 and induce apoptosis in human endothelial cells. *Nat Med* 2000;6:1183–6.
 42. Claman HN, Giorno RC, Seibold JR. Endothelial and fibroblastic activation in scleroderma: the myth of the "uninvolved skin." *Arthritis Rheum* 1991;34:1495–501.
 43. Stratton RJ, Coghlan JG, Pearson JD, Burns A, Sweny P, Abraham DJ, et al. Different patterns of endothelial cell activation in renal and pulmonary vascular disease in scleroderma. *QJM* 1998;91:561–6.
 44. Kraling BM, Jimenez SA, Sorger T, Maul GG. Isolation and characterization of microvascular endothelial cells from the adult human dermis and from skin biopsies of patients with systemic sclerosis. *Lab Invest* 1994;71:745–54.

45. Majewski S, Hunzelmann N, Johnson JP, Jung C, Mauch C, Ziegler-Heitbrock HW, et al. Expression of intercellular adhesion molecule-1 (ICAM-1) in the skin of patients with systemic sclerosis. *J Invest Dermatol* 1991;97:667-71.
46. Sollberg S, Peltonen J, Uitto J, Jimenez SA. Elevated expression of β_1 and β_2 integrins, intercellular adhesion molecule 1, and endothelial leukocyte adhesion molecule 1 in the skin of patients with systemic sclerosis of recent onset. *Arthritis Rheum* 1992;35:290-8.
47. Denton CP, Bickerstaff MC, Shiwen X, Carulli MT, Haskard DO, Dubois RM, et al. Serial circulating adhesion molecule levels reflect disease severity in systemic sclerosis. *Br J Rheumatol* 1995;34:1048-54.
48. Veale DJ, Kirk G, McLaren M, Belch JJ. Clinical implications of soluble intercellular adhesion molecule-1 levels in systemic sclerosis. *Br J Rheumatol* 1998;37:1227-8.
49. Abraham DJ, Varga J. Scleroderma: from cell and molecular mechanisms to disease models. *Trends Immunol* 2005;26:587-95.
50. Dignat-George F, Sampol J. Circulating endothelial cells in vascular disorders: new insights into an old concept. *Eur J Haematol* 2000;65:215-20.
51. Kang HJ, Kim HS, Zhang SY, Park W, Cho HJ, Koo BK, et al. Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: the MAGIC cell randomised clinical trial. *Lancet* 2004;363:751-6.
52. Vasa M, Fichtlscherer S, Adler K, Aicher A, Martin H, Zeiher AM, et al. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. *Circulation* 2001;103:2885-90.
53. Huang P, Li S, Han M, Xiao Z, Iang R, Hang ZC. Autologous transplantation of granulocyte colony-stimulating factor mobilized peripheral blood mononuclear cells improves critical limb ischemia in diabetes. *Diabetes Care* 2005;28:2155-60.