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Decreased numbers of endothelial progenitor cells in patients in the early stages of systemic sclerosis

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ABSTRACT

Introduction: Microangiopathy and endothelial dysfunction are present in the early stages of systemic sclerosis (SSc). Defective vasculogenesis mediated by bone marrow-derived endothelial progenitor cells (EPCs) might be involved in the vascular abnormalities found in SSc.
Objectives: To evaluate the circulating EPC levels and EPC subtypes via flow cytometry and early outgrowth colony-forming units (CFUs) in patients with SSc compared to healthy subjects.
Methods: Thirty-nine female SSc patients (30 in the early stages of SSc) and 44 age-matched healthy women were included. Peripheral blood EPCs were quantified using flow cytometry and by counting the early outgrowth CFUs.
Results: The EPCs quantified with flow cytometry and the CFU numbers were significantly lower in SSc patients than in control subjects (155.1 ± 95.1 vs. 241.3 ± 184.2 EPC/10^6 lymphomononuclear cells, $p = 0.011$; 15.4 ± 8.6 vs. 23.5 ± 10.9 CFU, $p < 0.001$; respectively), as well as in the group of patients in the early stages of SSc compared to the controls. Patients with digital ulcers had significantly higher CFU counts than those without ulcers ($p = 0.013$). Among patients with the scleroderma pattern on nailfold capillaroscopy, patients with the late pattern had significantly lower EPC levels than those with the early and active patterns ($p = 0.046$). There were no significant correlations of EPCs or CFU levels with RP duration.
Conclusions: The present study revealed decreased EPCs in SSc patients, including those with early disease onset. These findings suggest that defective vasculogenesis occurs in the early phases of the disease. Therefore, EPCs might be an important therapeutic target for the prevention of vascular complications in SSc patients.

Introduction

Systemic sclerosis (SSc) is a chronic autoimmune rheumatic disease that is characterized by fibrosis of the skin and internal organs. Vascular abnormalities characterized by endothelial damage and microvascular wall remodeling are among the major features that are involved in the pathogenesis of SSc (Flavahan et al., 2003; Gabrielli et al., 2009). Endothelial cell activation and apoptosis are present in the early stages of the disease and are followed by enhanced perivascular inflammatory infiltrate, progressive vessel narrowing, and capillary loss (Del Papa et al., 2006; Manetti et al., 2010). These vascular changes result in decreased blood flow, chronic tissue hypoxia and severe peripheral ischemic complications, such as digital ulcers and gangrene (Blaise et al., 2014). In addition to these abnormalities, SSc patients exhibit defective vascular repair and insufficient compensatory formation of new vessels (J.H.W. Distler et al., 2009).

The formation of new blood vessels following vascular injury essentially occurs via endothelium sprouting from preexisting endothelial cells (angiogenesis) and also from vasculogenesis, a process in which endothelial progenitor cells (EPCs) are mobilized from the bone marrow (Cipriani et al., 2007; Kuwana and Okazaki, 2012). EPCs have the ability to develop into fully mature endothelial cells and can target sites of endothelial injury to contribute to neovascularization (Asahara et al., 1997; Westerweel and Verhaar, 2009). Thus, reductions in the numbers and abnormal functioning of EPCs have been observed in several cardiovascular diseases and have been shown to play important roles in vascular injury and hypoxic conditions including SSc (J.H.W. Distler et al., 2009; Westerweel and Verhaar, 2009). Particularly in SSc, decreases in the numbers and impaired functioning of the EPCs are thought to play a role in the pathogenesis of the disease (Kuwana and Okazaki, 2012). Nonetheless, the numbers and functions of these cells in SSc remain to be matters of debate. Following the first report of Kuwana et al. (2004) that showed decreased levels of circulating EPCs in SSc patients, conflicting reports have been released in terms of the numbers and functions of EPCs in SSc (Allanore et al., 2007; Avouac et al., 2008b; Del Papa et al., 2004, 2006; Mok et al., 2010; Nevskaya et al., 2008; Zhu et al., 2008).

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EPCs are defined primarily by colony-forming assays that use culture-based methods or expression of cell-surface antigens including CD34, CD133 and vascular endothelial growth factor receptor type 2 (VEGFR2) as assayed by flow cytometry. Nonetheless, isolating and counting EPCs is not a standardized procedure, and the protocols used to quantify EPCs vary between studies (Kuwana and Okazaki, 2012). Recently, in an effort to standardize EPC research, the European League Against Rheumatism Scleroderma Trials and Research group (EUSTAR) proposed recommendations for the identification and measurement of EPCs (J. Distler et al., 2009).

Despite the important role of vascular regeneration and repair in the initial stages of SSc, few studies have evaluated the EPC levels of patients with recent disease. The aims of this study were to evaluate the numbers of circulating EPCs via flow cytometry analyses and early outgrowth EPC colony-forming unit assays in patients with SSc compared to a control group and to correlate the EPC levels with the clinical, laboratory and nailfold capillaroscopic features of SSc. A subgroup analysis of 30 patients with early stages of SSc was performed.

Patients and methods

Patients

Thirty-nine women with SSc who met the 2013 American College of Rheumatology/European League against Rheumatism (ACR/EULAR) classification criteria (van den Hoogen et al., 2013) were consecutively recruited in the Rheumatology Division of the Federal University of São Paulo. Thirty patients were in an early stage of the disease as defined as less than 3 years of disease for the diffuse cutaneous subset and less than 5 years for the limited cutaneous subset (Medsger and Steen, 1996). Only patients who experienced at least one Raynaud's phenomenon (RP) attack per day during the week before recruitment were included. The exclusion criteria were as follows: overlap with other connective tissue diseases, smoking, diabetes, pregnancy, dyslipidemia, liver disease, malignancies, and current use of drugs that might play roles in EPC mobilization, such as statins, endothelin receptor antagonists, mycophenolate and phosphodiesterase inhibitors. Forty-four healthy age-matched women were included as controls. This study was approved by the local ethics committee board of the Hospital of São Paulo (protocol number 1434/10). All patients provided their written informed consent to participate in the study including the collection of blood samples.

Clinical assessment

Data regarding the disease features and global clinical evaluations, including information about RP duration, disease duration (defined as the onset of the first non-Raynaud's symptom), modified Rodnan Skin Score (mRSS) (Rodnan et al., 1979), the presence of digital ulcers, arthritis, positivities for anti-centromere antibodies (ACA) and anti-topoisomerase I (anti-Scl-70) antibodies, interstitial lung disease, pulmonary arterial hypertension (defined by estimated pulmonary arterial systolic pressure > 40 mm Hg on Doppler echocardiography or by right-sided heart catheterization) and gastrointestinal involvement (esophageal, gastric and intestinal involvement), were collected from all subjects. The SSc patients were also classified into diffuse or limited cutaneous disease groups (LeRoy et al., 1988). Drug therapy data were collected from all individuals including the use of immunosuppressants, corticosteroids, vasodilators, and antiaggregants.

Additionally, all patients were instructed to record the number and duration of RP attacks during the week before the evaluation. The overall impact of the RP attacks was evaluated using the Raynaud's Condition Score (RCS), which is a daily self-assessment of RP activity on a 0–10 scale, as previously described (Merkel et al., 2002). The vascular domain of the Medsger Severity Disease Scale (Medsger et al., 1999) was used to evaluate the severity of peripheral vascular disease.

Panoramic widefield nailfold capillaroscopy (NFC) was performed using a stereomicroscope (Olympus — SZ40) at 10–25× magnification, as previously described (Andrade et al., 1990; Sekiyama et al., 2013). Briefly, the following parameters were determined in the eight fingers excluding thumbs: the number of capillaries/mm, the number of enlarged and giant capillary loops, the number of microhemorrhages, and the avascular score (Andrade et al., 1990; Maricq, 1981). The avascular score was determined semi-quantitatively on a scale of 0 to 3 (Lee et al., 1983). All of the parameters were recorded as the average obtained across all of the analyzed fingers. The patients who exhibited the scleroderma (SD) pattern were subdivided into three NFC pattern groups (i.e., early, active and late) as previously described (Cutolo et al., 2004).

Peripheral blood collection

Thirty milliliters of venous blood samples from all subjects was obtained on the same date as the clinical evaluation in the morning and processed as described below.

Flow cytometry analysis

The number of circulating EPCs was assessed with the flow cytometry method following the current recommendations (J. Distler et al., 2009). Ten milliliters of venous blood was collected from the peripheral forearm vein into an EDTA-containing tube and immediately transported to the laboratory for testing. First, peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density gradient centrifugation (Ficoll-Paque, GE Healthcare, UK). One million cells were counted using a Neubauer chamber and incubated with 4 μL of fluorescein-isothiocyanate (FITC)-conjugated CD34 (Southern Biotechnology Associates Inc., AL, USA), 4 μL of allophycocyanine (APC)-

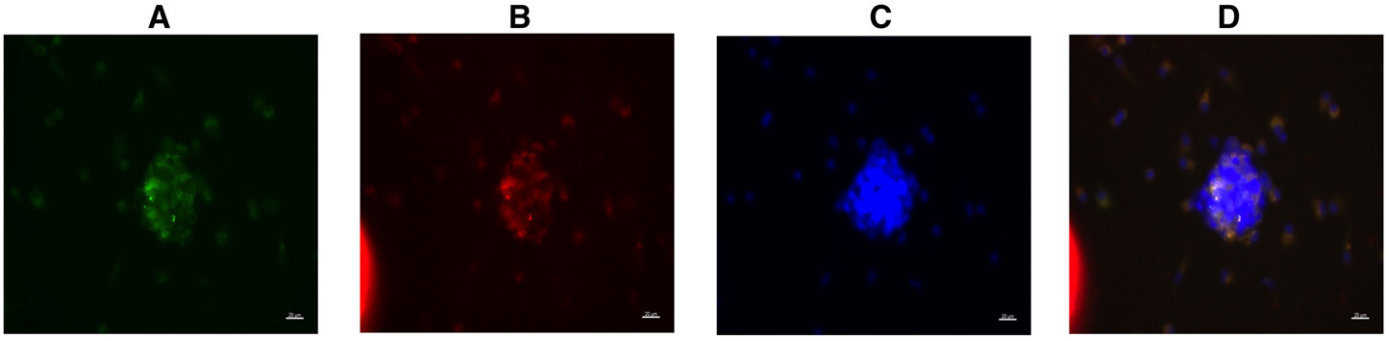

Fig. 1. Confirmation of the endothelial nature of the EPC colony-forming unit (CFU) assays obtained after 5 days. Cells from the colony positively stained for *Ulex europaeus* (green; A), Dil-labeled-acetylated LDL (red; B), and DAPI nuclear stain (blue; C). An overlay of the 3 colors confirms the endothelial nature of these cells (D).