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Increase in Circulating Endothelial Precursors by Atorvastatin in Patients With Systemic Sclerosis

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Objective. **To evaluate whether atorvastatin can increase bone marrow–derived circulating endothelial precursors (CEPs) and improve the vascular symptoms in patients with systemic sclerosis (SSc; scleroderma).**

Methods. **The study was designed as an open-label, prospective study involving 14 patients with SSc who received 10 mg/day of atorvastatin for 12 weeks and were followed up for the subsequent 4 weeks. CEPs were quantified at weeks 0 (pretreatment), 4, 8, 12 (during treatment), and 16 (posttreatment) by cell sorting followed by 3-color flow cytometry. Raynaud's phenomenon variables, global measures, and psychological scales as well as circulating angiogenic factors and endothelial activation/injury markers were serially assessed. The potential of CEPs to differentiate into mature endothelial cells was examined in cultures with angiogenic stimuli.**

Results. **None of the patients experienced an adverse event, but 1 dropped out because of an excessive decrease in serum total cholesterol. Atorvastatin treatment resulted in a 1.7- to 8.0-fold increase in CEPs from baseline levels ($P < 0.0001$), but the numbers returned to within baseline levels at posttreatment. However, 8 patients (62%) experienced a gradual decrease in the number of CEPs, even while taking atorvastatin. Variables indicating the extent of Raynaud's phenomenon improved significantly, and up-regulated levels of angiogenic factors and vascular endothelial activation/injury markers decreased significantly during atorvastatin treatment. These variables returned to within baseline levels after discontinuation of the drug. In contrast, atorvastatin failed to improve the in vitro maturation potential of CEPs.**

Conclusion. **The results of this pilot study suggest that atorvastatin treatment can increase CEPs and may be effective in improving Raynaud's phenomenon, even in SSc patients who have CEP dysfunction intrinsically.**

Systemic sclerosis (SSc; scleroderma) is a multiorgan disease characterized by excessive fibrosis and microvascular abnormalities (1). SSc vasculopathy mainly affects the small arteries and causes reduced blood flow and tissue ischemia, which can lead to Raynaud's phenomenon, digital ulcers, and gangrene (1). A primary mechanism for the vascular involvement in patients with SSc is thought to be the enhancement of vascular injury occurring as a result of the inflammation/immunopathologic process and the ischemia-reperfusion reaction. However, we have recently proposed another theory, that insufficient mechanisms of vascular repair, due to defective vasculogenesis, contribute to the pathogenic process (2).

Vasculogenesis requires the recruitment of bone marrow–derived circulating endothelial precursors (CEPs) to form the blood vessels (3). This process has been believed to occur exclusively during blood vessel development, but recent accumulating evidence indicates that CEPs contribute to vascular healing in response to vascular injury or ischemia in adults, by homing to the site of injury and then working in concert with existing mature endothelial cells (4,5). However, in healthy adults, CEPs are a very rare circulating cell population, being present in only ~1 of 10^6 leukocytes (2,4).

Human CEPs can be identified by a characteristic

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surface phenotype that is positive for CD34, CD133, and vascular endothelial growth factor (VEGF) receptor type 2 (VEGFR-2) (4). We have developed assay systems to evaluate the absolute number of CEPs and their maturation potential, and used them to examine the quantity and function of CEPs in patients with SSc (2). In these patients, we observed a markedly reduced number of CEPs, and the CEPs had an impaired maturation potential in response to angiogenic factors, in comparison with healthy controls.

This finding led us to propose that strategies to increase the levels of CEPs have therapeutic potential for SSc vasculopathy. One drug family with this potential is the 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase inhibitors, or statins. In addition to their lipid-lowering effect, statins have various potential activities, including the suppression of inflammation, improvement of vascular function, and inhibition of smooth muscle proliferation; these multiple functions are called pleiotropic effects (6). Statins have recently been shown to stimulate CEP kinetics and increase the number of CEPs in the circulation of patients with stable coronary artery disease (7). We therefore conducted the present open-label, prospective study to evaluate whether statins have the capacity to increase CEPs and improve vascular symptoms in patients with SSc.

PATIENTS AND METHODS

Study design. The present study, a single-center, open-label, prospective study, involved 14 patients with SSc at Keio University Hospital in Tokyo, Japan, conducted from June 2004 to March 2005. All patients fulfilled the American College of Rheumatology preliminary classification criteria for SSc (8). Patients were enrolled regardless of whether SSc was of the diffuse or limited cutaneous form, and regardless of the serum total cholesterol level. The exclusion criteria included age <18 years, pregnancy or lactation, a history of potential adverse effects associated with statins, current treatment with statins or drugs known to interact with statins (e.g., fibrates and cyclosporine), the presence of another well-defined rheumatic disease except Sjögren's syndrome, serious organ involvement (e.g., chronic respiratory failure due to pulmonary interstitial fibrosis or uncontrolled malabsorption), or other debilitating illness (e.g., cancer).

All of the study patients received atorvastatin at a dosage of 10 mg once a day for 12 weeks, and were then followed up for the subsequent 4 weeks. Atorvastatin was started in the fall (September–November) when the ambient temperature in Tokyo declines. Peripheral blood samples were obtained from all patients at 5 time points: week 0 (pretreatment), weeks 4, 8, and 12 (during treatment), and week 16 (posttreatment). All patients completed a daily diary to track the occurrence and rate the severity of Raynaud's phenomenon attacks at weeks 0, 4, 8, 12, and 16, and completed questionnaires for the assessment of functional status at weeks 0, 12, and 16. The patients were allowed to continue their other therapies during the study period, provided that the drug dosages were maintained at a constant level until the study was completed. The study protocol conformed to the ethics principles of the World Medical Association Declaration of Helsinki as reflected in a priori approval from our institutional review board, and written informed consent was obtained from each patient.

Measurement of functional status in relation to Raynaud's phenomenon. The activity, disability, pain, and psychological impact associated with Raynaud's phenomenon were evaluated using a proposed core set of outcome measures for studying Raynaud's phenomenon in SSc patients (9). These measures included the Raynaud's Condition Score (RCS; a daily self-assessment of Raynaud's phenomenon activity using a 0–10 ordinal scale), the Health Assessment Questionnaire Disability Index (scale 0–3) (10), visual analog scales (VAS) for Raynaud's phenomenon, digital ulcers, pain, and overall disease (scales of 0–3), a VAS for the physician's global assessment of health (scale 0–3), and the mood and tension scales of the Arthritis Impact Measurement Scales 2 (scale 0–10) (11). The RCS values over the 2-week period prior to each assessment visit were averaged and expressed as the mean ± SD.

Antinuclear antibody analysis. SSc-related antinuclear antibodies were identified using indirect immunofluorescence and immunoprecipitation assays, as described previously (12).

Quantification of CEPs. The absolute numbers of CEPs and overall proportion of CD34+ cells in 20 ml of peripheral blood were quantified as described previously (2). Briefly, samples partially enriched in CD34+ cells from peripheral blood mononuclear cells by a magnetic-activated cell sorter (MACS) immunomagnetic technique (Miltenyi Biotech, Bergisch Gladbach, Germany) were subjected to 3-color flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) with a fluorescein isothiocyanate (FITC)–conjugated anti-CD34 monoclonal antibody (mAb), phycoerythrin-conjugated anti-CD133 mAb (Miltenyi Biotech), and anti–VEGFR-2 mAb (Sigma, St. Louis, MO) in combination with biotin-conjugated goat anti-mouse IgG $F(ab')_2$ and streptavidin-PC5 (Beckman Coulter, Fullerton, CA). The expression of CD133 and VEGFR-2 was evaluated on gated CD34+ cells, and the total number of viable cells in the CD34+ cell–enriched fraction was determined by its ratio to the FlowCount microbeads (Beckman Coulter). All procedures were performed by the same operator (YO), who was blinded to the sample identity.

Circulating levels of angiogenic factors and endothelial activation/injury markers. The levels of VEGF, soluble vascular cell adhesion molecule 1 (VCAM-1), and soluble E-selectin in heparinized platelet-poor plasma and the levels of basic fibroblast growth factor (bFGF) in serum were measured using specific enzyme-linked immunosorbent assay kits (Quantikine; R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

In vitro maturation of CEPs. The potential of CEPs to differentiate into mature endothelial cells in response to angiogenic stimuli was evaluated as described previously (2). Briefly, CD133+ cells separated from peripheral blood mononuclear cells by MACS were coplated with CD133− cells on

fibronectin-coated chamber slides and cultured in endothelial cell basal medium 2 (EBM-2) supplemented with EBM-2 MV SingleQuots (Clonetics, San Diego, CA) for 5 days. The cells were fixed and incubated with mouse anti–VEGFR-2 or anti–von Willebrand factor (vWF) mAb (Dako, Carpinteria, CA) followed by incubation with AlexaFluor 568 mouse-specific IgG (Molecular Probes, Eugene, OR) and then with FITC-conjugated anti-CD45 mAb (Dako). The stained cells were examined with a confocal laser fluorescence microscope (LSM5 Pascal; Carl Zeiss, Göttingen, Germany). The proportion of mature endothelial cells derived from CEPs in this in vitro maturation process was calculated as the ratio of CD45−,vWF+ cells to CD45−,VEGFR-2+ cells, with results expressed as a percentage.

Statistical analysis. Frequencies between 2 groups were tested for statistical significance using Fisher's 2-tailed exact test. All continuous values are expressed as the mean ± SD. Changes in the absolute values at different time points from baseline (at week 0) were compared by repeated-measures analysis of variance. When the P value for this overall comparison was considered significant ($P < 0.05$), post-hoc pairwise comparisons were performed using Dunnett's test.

RESULTS

Baseline characteristics. The study group comprised 14 female patients with SSc, between the ages of 36 years and 75 years (mean ± SD age 57.4 ± 11.0 years). The disease duration from the first symptom attributable to SSc, including Raynaud's phenomenon, ranged from 13 months to 261 months (mean ± SD 121 ± 78 months). Seven patients each had diffuse SSc and limited cutaneous SSc. Assays for the antinuclear antibody status revealed 6 patients with anti–Scl-70/topoisomerase I, 5 with anticentromere, 2 with anti–RNA polymerase III, and 1 with anti–U1 RNP antibody. The modified Rodnan skin thickness score (13) ranged from 4 to 28 (mean ± SD 10.1 ± 8.0). All patients had Raynaud's phenomenon, and 2 had ulcers on multiple digital tips at the time of entry. Four patients were receiving low-dose prednisolone (<10 mg daily), but none of them were receiving an immunosuppressive agent or D-penicillamine. All patients were receiving at least 1 of the following vasoactive or antioxidant agents: oral prostacyclin, calcium channel blocker (e.g., amlodipine), low-dose aspirin, and vitamin E. Only 3 patients had hypercholesteremia (total cholesterol level >220 mg/dl).

Adverse events. All but 1 patient completed the 12 weeks of atorvastatin treatment and continued to receive stable doses of other medications throughout the

Table 1. Serial measurements of CEP numbers and other variables during atorvastatin therapy*

	Week 0 (pretreatment)	Week 4	Week 8	Week 12	Week 16 (posttreatment)	Overall P
CD34+ cells (× 10^3/20 ml peripheral blood)	7.8 ± 5.0	10.5 ± 7.3	11.2 ± 7.6	12.6 ± 9.2†	7.8 ± 4.7	0.03
CEP, /20 ml peripheral blood	132 ± 78	300 ± 188‡	295 ± 123‡	308 ± 208‡	111 ± 55	<0.0001
Raynaud's phenomenon variables						
Raynaud's Condition Score	4.41 ± 1.45	3.64 ± 1.46	3.63 ± 1.82	2.95 ± 1.60‡	3.39 ± 1.58	0.04
Patient's assessment by VAS	1.22 ± 0.73	NT	NT	0.73 ± 0.38†	1.03 ± 0.68	0.03
Psychological scales						
AIMS2 tension scale	2.46 ± 1.91	NT	NT	2.35 ± 1.73	2.49 ± 2.03	NS
AIMS2 mood scale	2.08 ± 1.51	NT	NT	1.69 ± 1.18	2.25 ± 1.86	NS
Disability and global measures						
HAQ DI	0.35 ± 0.29	NT	NT	0.35 ± 0.32	0.37 ± 0.31	NS
Patient's global assessment by VAS	1.10 ± 0.59	NT	NT	0.88 ± 0.73	0.91 ± 0.68	NS
Physician's global assessment by VAS	1.21 ± 0.52	NT	NT	0.89 ± 0.47	0.89 ± 0.47	NS
Pain scale, patient's assessment by VAS	0.55 ± 0.64	NT	NT	0.60 ± 0.68	0.51 ± 0.53	NS
Modified Rodnan total skin thickness score (scale 0–51)	8.8 ± 6.3	NT	NT	8.8 ± 6.4	8.6 ± 6.1	NS
Total cholesterol, mg/dl§	205 ± 36	155 ± 26‡	157 ± 23‡	158 ± 27‡	195 ± 26	<0.0001
Angiogenic factors						
VEGF, pg/ml	29.7 ± 9.1	29.6 ± 10.1	29.4 ± 8.7	25.8 ± 8.7†	26.3 ± 7.9	0.02
bFGF, pg/ml	14.3 ± 4.4	10.0 ± 5.4‡	10.1 ± 4.4‡	9.7 ± 5.7‡	12.8 ± 4.8	<0.0001
Endothelial activation/injury markers						
Soluble VCAM-1, ng/ml	594.9 ± 189.0	545.7 ± 192.7‡	496.5 ± 172.7‡	517.3 ± 169.5‡	556.1 ± 192.1‡	<0.0001
Soluble E-selectin, ng/ml	44.7 ± 16.0	42.2 ± 14.4	40.3 ± 13.2‡	42.9 ± 15.0	42.9 ± 14.6	0.007

* Values are the mean ± SD. CEP = circulating endothelial precursor; VAS = visual analog scale; NT = not tested; AIMS2 = Arthritis Impact Measurement Scales 2; NS = not significant; HAQ DI = Health Assessment Questionnaire Disability Index; VEGF = vascular endothelial growth factor; bFGF = basic fibroblast growth factor; VCAM-1 = vascular cell adhesion molecule 1.
† $P < 0.05$ versus week 0.
‡ $P < 0.01$ versus week 0.
§ One patient dropped out at week 8, due to an excessive decrease in the total cholesterol level to <100 mg/dl.

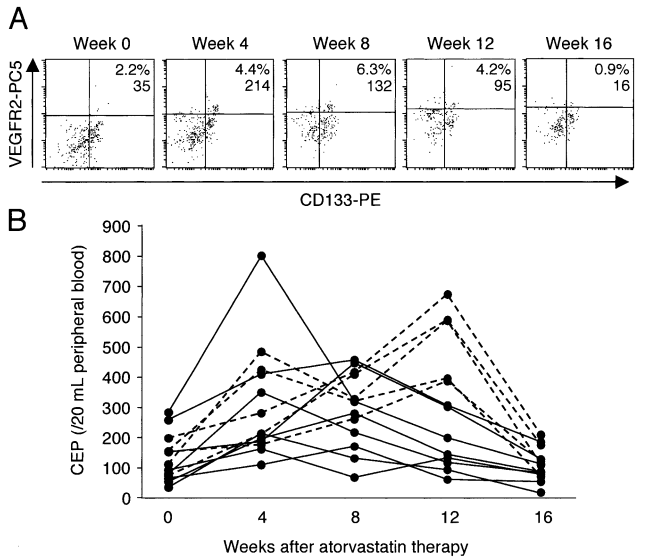

Figure 1. Effects of atorvastatin on the levels of circulating endothelial precursors (CEPs) in the peripheral blood of patients with systemic sclerosis (SSc). **A,** For serial measurements of CEPs by flow cytometry (shown in a representative SSc patient), the CD34+ cell–enriched fraction was stained with anti-CD34 (fluorescein isothiocyanate), anti-CD133 (phycoerythrin [PE]), and anti–vascular endothelial growth factor receptor type 2 (VEGFR-2) (VEGFR2-PC5) monoclonal antibody. CD34+ cells were gated and analyzed for the expression of CD133 and VEGFR-2. The upper-right section of the individual dot-plot images indicates the CD34+,CD133+,VEGFR-2+ CEPs. Values are the proportion of CEPs in the gated CD34+ cells and absolute number of CEPs in 20 ml of peripheral blood. **B,** Serial changes in absolute CEP numbers in 20 ml of peripheral blood were determined in peripheral blood samples from 13 SSc patients at 5 time points: week 0 (pretreatment), weeks 4, 8, and 12 (during atorvastatin treatment), and week 16 (posttreatment). CEP changes at the different time points from baseline were statistically significant ($P < 0.0001$) by repeated-measures analysis of variance, and CEP levels at weeks 4, 8, and 12 were significantly different from week 0 levels by post-hoc pairwise comparisons. The patients were divided into 2 groups based on the time course of the CEP response: solid lines indicate patients in group 1, in whom the number of CEPs peaked at week 4 or week 8 and decreased thereafter, even during atorvastatin treatment (n = 8), while broken lines indicate those in group 2, in whom the number of CEPs gradually increased during treatment (n = 5).

study. None of the patients experienced any adverse events during atorvastatin treatment, but 1 patient dropped out at week 8 because of an excessive decrease in total cholesterol level (to <100 mg/dl). After exclusion of this patient, the total cholesterol level in the remaining 13 patients with SSc was significantly reduced during atorvastatin treatment (reductions of 24%, 23%, and 23% at weeks 4, 8, and 12, respectively; $P < 0.0001$) but returned to within baseline levels at week 16 (Table 1).

Effects on CEPs. Serial flow cytometric analyses for CD133 and VEGFR-2 on gated CD34+ cells for a representative SSc patient are shown in Figure 1A. CEPs, identified as cells positive for both CD133 and VEGFR-2, were scarcely detected at pretreatment (week 0) and posttreatment (week 16), but were clearly visible during atorvastatin treatment (weeks 4, 8, and 12). The changes in the absolute number of CEPs before and after atorvastatin treatment are illustrated in Figure 1B. Treatment with atorvastatin resulted in a 1.7- to 8.0-fold increase (mean ± SD increase 3.8 ± 1.9) in the CEP number from baseline (mean ± SD 132 ± 78), but the CEP numbers (mean ± SD 300 ± 188, 295 ± 123, and 308 ± 208 at weeks 4, 8, and 12, respectively) did not reach the levels reported in healthy individuals (mean 1,074) (2). The CEP numbers returned to within baseline levels (mean ± SD 111 ± 55) after the cessation of atorvastatin at week 16 in all patients. The increase in CEP numbers from baseline at weeks 4, 8, and 12 was statistically significant ($P < 0.0001$) (Table 1).

The overall number of circulating CD34+ cells also increased during atorvastatin treatment ($P = 0.03$). Interestingly, there were 2 patterns of CEP response. In one pattern, CEP numbers peaked at week 4 or week 8 and were reduced thereafter, even during atorvastatin treatment (n = 8; group 1), and in the other response pattern, CEP numbers gradually increased during treatment (n = 5; group 2). When the clinical features were compared between these 2 groups, 75% of the patients in group 1 experienced digital ulcers at least once during the course of the disease, whereas none of the group 2 patients developed digital ulcers ever during the course of the disease ($P = 0.02$). Other characteristics distinguishing group 1, but without statistical significance, included a predominance of diffuse cutaneous SSc (63% versus 20% in group 2) and active digital ulcers at entry (25% versus 0% in group 2), but there was no difference in age at examination, disease duration, or RCS at entry between these 2 groups.

Effects on Raynaud's phenomenon activity. The serial changes in the Raynaud's phenomenon variables, psychological scores, disability and global measures, and pain scores during the study period are summarized in Table 1. The manifestations of Raynaud's phenomenon improved during atorvastatin treatment, with significant reductions in the RCS (−1.46 in mean daily score and 33% reduction in mean score at week 12; $P = 0.04$) and the patient's assessment by VAS (−0.49 in mean daily score and 40% reduction in mean score at week 12; $P = 0.03$). These variables tended to worsen after the discontinuation of atorvastatin. There was no difference in the

changes in these variables between CEP response groups 1 and 2. Although there were trends toward improvement in the mood scale and in the patient's and physician's global assessments by VAS at week 12, these changes were not statistically significant. None of the patients, including the 2 with digital ulcers at the time of entry, developed new digital ulcers during treatment with atorvastatin, but 1 patient developed new digital ulcers 2 weeks after the discontinuation of atorvastatin.

Effects on angiogenic factors and endothelial activation/injury markers. The circulating levels of angiogenic factors (VEGF and bFGF) and endothelial activation/injury markers (soluble VCAM-1 and soluble E-selectin) were measured before and after atorvastatin treatment (Table 1). The levels of these molecules are known to be elevated in the circulation of patients with SSc as compared with healthy controls (2,14). The levels of all of these circulating angiogenic factors and soluble endothelial activation/injury markers were significantly reduced during atorvastatin treatment as compared with baseline levels, but all returned to within baseline levels after the discontinuation of atorvastatin. There was no difference in the changes in these circulating markers between the 2 different CEP response groups.

Maturation potential of CEPs. The capacity of CEPs to maturate in response to in vitro angiogenic stimulation was evaluated at weeks 0 and 12 in 5 patients with SSc (2 in CEP response group 1, and 3 in group 2). The number of CEPs at week 12 was greater than that at week 0 in 4 of the patients, but was lower in 1 patient (in group 1). The maturation potential of CEPs was impaired in all 5 patients at baseline, and was not improved after the 12-week treatment with atorvastatin (mean ± SD 15.3 ± 4.9% at week 0 versus 17.9 ± 5.1% at week 12).

DISCUSSION

In this pilot study, short-term treatment with atorvastatin was associated with an increase in the number of CEPs in SSc patients. In addition, significant improvement in the ratings of Raynaud's phenomenon and reductions in the up-regulated levels of angiogenic factors and vascular endothelial activation/injury markers were observed during treatment with atorvastatin. Most of these variables returned to within baseline levels 4 weeks after the discontinuation of atorvastatin, confirming a link between these changes and atorvastatin therapy. The beneficial effects observed during atorvastatin treatment could be explained by the recruitment of CEPs into the periphery and the repair of injured endothelium, since statins have been shown to increase the number of CEPs and to promote vasculogenesis in vivo in animal models of ischemia (15–18). However, it is also possible that the observed clinical changes were mediated through other effects of statins, such as anti-inflammation mechanisms and the improvement of mature endothelial function (6).

A limitation of this study is that clinical assessment of Raynaud's phenomenon was carried out using the RCS and VAS rating in the setting of an open-label study; nevertheless, these measures are shown to be reliable for measuring Raynaud's phenomenon activity in SSc patients (9). This study would have benefited from inclusion of objective measures of small-vessel blood flow, such as pulse-wave analysis. Although the results of this preliminary study are encouraging, further multicenter, placebo-controlled trials involving a large number of SSc patients are necessary to confirm the clinical benefit of statins in SSc patients.

Despite their increase, the numbers of CEPs during atorvastatin treatment did not reach the level seen in healthy individuals, and the statin-induced CEP response was transient in many of the patients with SSc, especially those with antecedent digital ulcers. In addition, when a combination of angiogenic stimuli was used to induce CEP maturation, the statin failed to improve the impaired maturation potential of the CEPs. These observations indicate that although atorvastatin is certainly capable of improving CEP dysfunction in SSc patients, its effects are limited.

The mechanism causing the reduced numbers of CEPs in SSc patients is currently unknown, but the elevated level of circulating VEGF, which is known to be a critical mediator of CEP recruitment to the periphery (19), strongly suggests that CEPs and their stem cells in the bone marrow do not respond adequately to angiogenic stimuli, and VEGF and other angiogenic factors are up-regulated in compensation for this defect. The proposed mechanisms by which statins enhance the CEP numbers include increasing their proliferation and mobilization, and preventing CEP senescence and apoptosis within the bone marrow (15–18,20). The statins' ability to increase CEP numbers mimics the effects of VEGF, but is independent of the mechansim of action of VEGF (16).

It is therefore likely that CEPs and/or their stem cells in SSc patients are functionally altered and are intrinsically hyporesponsive to both VEGF and statins. In this regard, it would be interesting to investigate the phosphatidylinositol 3-kinase/Akt pathway in the CEPs from SSc patients, since the activation of this signaling

pathway is one of the critical events required for the increase in CEP levels induced by both VEGF and statins (15–17). Alternatively, continuous endothelial injury might lead to the eventual depletion of CEPs, as has been suggested to occur in patients with multiple risk factors for atherosclerosis (21). The transient CEP response induced by atorvastatin that was observed in patients with antecedent digital ulcers (group 1) might be consistent with this scenario.

Our study suggests that stimulation of CEP mobilization and/or differentiation may provide a novel therapeutic strategy for improving peripheral vascular disease in SSc patients. In this regard, in addition to statins, the administration of drugs that exert potent stimulatory effects on CEP kinetics, such as granulocyte–macrophage colony-stimulating factor (22) and granulocyte colony-stimulating factor (4), could augment vasculogenesis as a therapeutic intervention for ischemic complications in patients with SSc.

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