Simvastatin Reduces Endothelial Activation and Damage But Is Partially Ineffective in Inducing Endothelial Repair in Systemic Sclerosis

NICOLETTA DEL PAPA, MICHELA CORTIANA, CLAUDIO VITALI, ILARIA SILVESTRIS, WANDA MAGLIONE, DENISE P. COMINA, TIZIANO LUCCHI, and AGOSTINO CORTELEZZI

ABSTRACT. ***Objective.*** To investigate whether statins may improve endothelial function in systemic sclerosis (SSc) by evaluating the effects of simvastatin on vasculogenesis [indicated by the expansion of circulating endothelial progenitor cells (EPC)] and the markers of vascular injury in the peripheral blood of patients with SSc.

Methods. Twenty SSc patients with normal cholesterol concentrations and 20 hypercholesterolemic subjects were allocated to receive 20 mg/day simvastatin for 12 weeks. Peripheral blood samples were collected before and 12 weeks after initiation of treatment, and 4 weeks after discontinuation. Five-parameter, 3-color flow cytometry was performed with a FacScan to enumerate EPC and mature circulating endothelial cells (CEC). Levels of soluble E-selectin, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, interleukin 6, and endothelin-1 were assessed by commercial ELISA.

Results. Simvastatin treatment significantly increased EPC in the hypercholesterolemic group, but failed to improve the EPC levels in the SSc patients, mainly in patients with late disease. Baseline levels of CEC were significantly higher in SSc patients compared with controls and at the end of the treatment they were significantly decreased. Regarding other markers of endothelial activation, we found that all the cytokine levels decreased in a statistically significant manner in the treated patients.

Conclusion. Treatment with simvastatin results in rapid and significant improvement of measures of endothelial activation, suggesting a potential role of statins in the treatment of peripheral vascular disease in SSc. The lack of effect on increase of EPC confirms our previous findings of a defective endothelial stem cell recruitment in the bone marrow of SSc patients. This could indicate that the potential effectiveness of statins in SSc could mainly be ascribed to their effectiveness in modulating endothelial activation mechanisms. (First Release June 1 2008; J Rheumatol 2008;35:1323–8)

Key Indexing Terms:
SIMVASTATIN ENDOTHELIAL ACTIVATION SYSTEMIC SCLEROSIS
ENDOTHELIAL PROGENITORS VASCULOGENESIS

Endothelial dysfunction and structural vessel-wall abnormalities are an early and crucial feature in the pathogenesis of systemic sclerosis (SSc)[1]. Vasculopathy involves microcirculation severely affecting blood flow with local platelet aggregation and thrombosis, resulting in further endothelial injury and chronic tissue ischemia.

Some studies have focused on the hypothesis that scleroderma vasculopathy is in part or largely the result of a failure in the mechanisms of vascular repair that are effective in ischemic conditions[2,3]. A growing body of evidence in experimental and clinical studies indicates that bone marrow-derived endothelial progenitor cells (EPC) participate in vascular homeostasis and make an important contribution in improving neovascularization after ischemia. In this context, different studies found that patients with SSc have, particularly in the late stages of the disease, fewer circulating EPC than healthy controls and the function of these cells is impaired[2-4]. These findings have been related recently to a complex impairment in the bone marrow microenvironment involving both endothelial and stromal progenitors[3].

Based on these observations, new therapeutic strategy for scleroderma vasculopathy could consider drugs capable of

From the Department of Rheumatology, G. Pini Hospital, Milano; Ematologia 1, Ospedale Maggiore IRCCS, Università degli Studi Milano, Milano; Department of Internal Medicine and Rheumatology, Ospedale Villamarina, Piombino; Unità Operativa di Geriatria, Ospedale Maggiore IRCCS, Milano; and Department of Haematology, Ospedale Policlinico IRCCS, Milano, Italy.

N. Del Papa, MD, Department of Rheumatology, G. Pini Hospital; M. Cortiana, PhD, Ematologia 1, Ospedale Maggiore IRCCS, Università degli Studi Milano; C. Vitali, MD, Department of Internal Medicine and Rheumatology, Ospedale Villamarina; I. Silvestris, PhD, Ematologia 1, Ospedale Maggiore IRCCS, Università degli Studi Milano; W. Maglione, MD; D.P. Comina, MD, Department of Rheumatology, G. Pini Hospital; T. Lucchi, MD, Unità Operativa di Geriatria, Ospedale Maggiore IRCCS; A. Cortelezzi, MD, Department of Haematology, Ospedale Policlinico IRCCS.

Address reprint requests to Dr. N. Del Papa, Department of Rheumatology, G. Pini Hospital, Via Pini 9, 20122 Milano, Italy. E-mail: delpapa@gpini.it

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inducing mobilization and migration of EPC to sites of ischemia. Among these, statins have been shown to have proangiogenic actions by promoting mobilization and differentiation of bone marrow EPC. In addition, studies showed that functional improvement and increased homing capacity of EPC, induced by statin treatment, might reverse the impaired functional regeneration capacities in patients with coronary artery disease and hypercholesterolemia[5-7]. A recent study by Kuwana, *et al* suggests that atorvastatin treatment can increase the number EPC, but fails to improve their impaired maturation potential in patients with SSc[8].

We investigated whether statins may be useful for endothelial repair in SSc by evaluating the effects of simvastatin on the presence of EPC. The potential effects of this drug in limiting the endothelial cell activation and the consequent damage was assessed by measuring some markers of endothelial activation and damage, such as the number of mature circulating endothelial cells (CEC), and the levels of some endothelial-specific adhesion molecules and cytokines, respectively.

MATERIALS AND METHODS

Patients. Twenty consecutive normocholesterolemic women with limited SSc (lSSc) were consecutively recruited for this open-label, prospective study. All patients fulfilled the American College of Rheumatology criteria for SSc[9] and were classified as having limited cutaneous SSc on the basis of the criteria of LeRoy, *et al*[10]. Clinical assessment of the study population was performed according to described criteria[11]. In particular, skin involvement was assessed by a modified Rodnan score. The occurrence of pulmonary involvement was defined by pulmonary hypertension and/or bibasilar fibrosis at standard chest radiograph and/or restrictive lung disease. Pulmonary function was assessed by vital capacity, measured by a dry spirometer, and by carbon monoxide-diffusing capacity (DLCO), measured by the single-breath method, both expressed as percentages. Pulmonary artery pressure was detected by echocolor Doppler cardiography.

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 scleroderma, dyspnea, and/or dysphagia). Disease stages were defined as suggested by Medsger and Steen[12]. In particular, disease duration had to be < 5 years and > 5 years to define a patient as having early or intermediate/late SSc, respectively.

Patients were allowed to continue previous therapies during the study period, provided drug dosages were maintained at a constant level until the study was completed. The following exclusion criteria were applied: pregnancy or lactation, a history of potential adverse effects associated with statins, current therapy with statins or drugs known to interact with statins, treatments with anticoagulants, prednisone > 7.5 mg/day, cyclophosphamide during the previous 3 months, presence of diabetes mellitus, hypertension and current tobacco use. The LDL cholesterol serum levels ranged from 57 to 207 mg/dl at the time of inclusion into the study. The age-matched control group consisted of 20 women with hypercholesterolemia (total serum cholesterol > 200 mg/dl). No patient had previously been treated with a statin.

All the SSc patients and controls received 20 mg of simvastatin per day over 12 weeks. Peripheral blood samples were collected at baseline (before starting statin therapy) as well as at Week 12 (immediately before stopping therapy) and at Week 16 (4 weeks after discontinuation of therapy) to measure EPC and CEC counts, serum cholesterol levels, and soluble plasma endothelial markers.

The study was approved by the local review board and ethics committee, and all patients gave their written informed consent.

Detection of EPC and CEC by flow cytometry. Total EPC and CEC were enumerated as described[3,4]. Briefly, CEC were defined as negative for CD45 and positive for CD31 and P1H12. The subset of stem endothelial precursor cells was identified in peripheral blood by the surface expression of CD34, VEGF2R (KDR), and CD133, a stem cell marker that is not expressed by mature endothelial cells. Five-parameter, 3-color flow cytometry was performed with a FacScan flow cytometer with a 15-mW argon laser (excitation at 488 mm; Becton Dickinson, San Jose, CA, USA) and data were analyzed with CellQuest software (Becton Dickinson).

Plasma cytokine levels. Plasma concentrations of soluble E-selectin, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), interleukin 6 (IL-6), and endothelin-1 (ET-1) were assessed by commercial ELISA (R&D Systems, Minneapolis, MN, USA) and calculated using a standard curve generated with specific standards, according to the manufacturer's recommendations.

We had decided not to evaluate cytokine levels in the control group since these data would not add to the knowledge of the pleiotropic effects of statin therapy in hypercholesterolemic patients[13].

Statistical analysis. Data are expressed as the mean ± SE. To assess the effect of therapy, pre- and post-treatment values of the biochemical variables were compared using Student's paired t-test.

RESULTS

Baseline clinical variables. The study included 20 women with a median age of 59 years (range 28–65 years). The median disease duration was 8 years (range 1–28 years). On the basis of Medsger and Steen's criteria[12], 9 (45%) patients had early disease and 11 (55%) patients had late disease. All the patients reported the presence of Raynaud's phenomenon. Cutaneous ulcers were found in 9 patients at time of entry to the study. All patients had a modified Rodnan score < 14. With respect to pulmonary involvement, 7 of 20 patients had no pulmonary involvement, 8 had primary pulmonary hypertension, and 5 had an interstitial lung disease. With regard to autoantibody profile, 14 patients were positive for anticentromere antibodies and 6 for antinuclear antibodies.

All patients were treated with intravenous monthly prostacyclin, daily calcium channel blockers, and/or angiotensin-converting enzyme inhibitors.

Adverse events. All the patients completed the study and there were no adverse reactions or events throughout the period of treatment with simvastatin.

Effect of simvastatin on EPC. As expected on the basis of previous studies[5-7,14], treatment with 20 mg simvastatin per day over a period of 12 weeks was associated with a significant increase in the number of circulating EPC in patients with hypercholesterolemia (1.99 ± 1.22 and 8.88 ± 2.05 EPC/ml at baseline and 12 weeks, respectively; p = 0.03). In contrast, measurement of circulating EPC in SSc patients receiving statin treatment over the same period revealed essentially identical values (3.17 ± 0.58 and 4.52 ± 0.80 cells/ml at baseline and 12 weeks, respectively; p = nonsignificant; Figure 1). Nevertheless, when the patients with SSc were stratified on the basis of disease duration, the number of circulating EPC was significantly increased in those with recent-onset disease (from 3.96 ± 0.94 to 6.90 ±

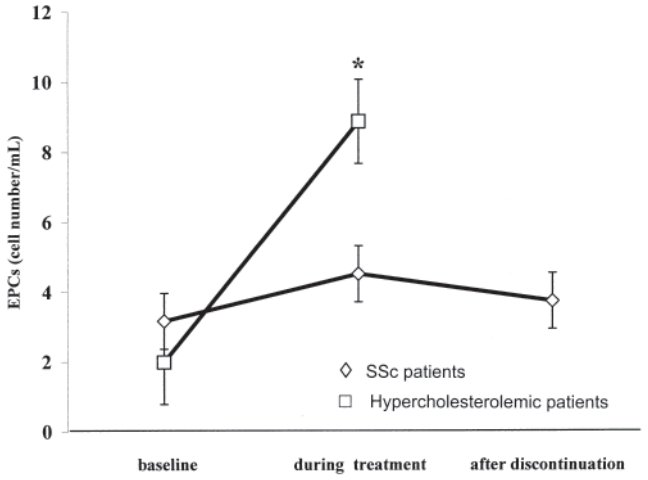

Figure 1. Flow cytometry evaluation of circulating endothelial progenitor cells (EPC) in peripheral blood of patients with SSc and in the hypercholesterolemic control group, at baseline, at the end, and after therapy. Values are mean and SE. *p = 0.03 for hypercholesterolemic patients at baseline versus the same group at the end of statin treatment.

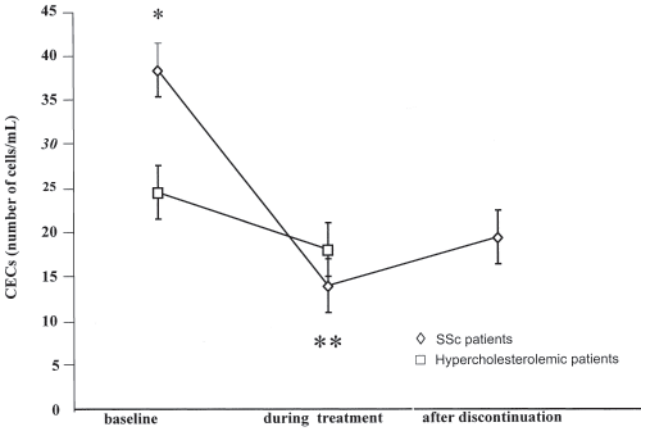

Figure 3. Flow cytometry evaluation of circulating endothelial cells (CEC) in peripheral blood of patients with SSc and in the hypercholesterolemic control group, at baseline, at the end, and after therapy. Values are mean and SE. *p = 0.04 for CEC levels in SSc versus the hypercholesterolemic group, and **p = 0.01 for SSc before versus after statin therapy.

1.55 cells/ml; p = 0.04) compared with those with late disease (from 2.38 ± 0.64 to 2.24 ± 0.62 cells/ml; p = nonsignificant; Figure 2). However, the absolute increase of EPC observed even in patients with early disease was strongly reduced compared to the hypercholesterolemic controls (p = 0.04). In addition, the number of peripheral EPC returned to baseline values in patients with early SSc after statin discontinuation, (data not shown), while no changes in EPC levels were observed in the late-disease group.

Effect of simvastatin on CEC. The baseline number of circulating mature endothelial cells was higher in patients with SSc compared with the control hypercholesterolemic group (38 ± 5 and 24.2 ± 5 CEC/ml; p = 0.04).

As illustrated in Figure 3, simvastatin treatment significantly decreased the number of CEC in the peripheral blood of SSc patients (from 38 ± 5 to 13.8 ± 4 CEC/ml; p = 0.01). In contrast, the therapy did not affect the total number of CEC in the control group (24.2 ± 5 vs 17.7 ± 3.7 CEC/ml after 12 weeks; p = nonsignificant). CEC counts in patients tended to increase after discontinuation (18.8 ± 4.7), but the changes mantained statistical significance in comparison with baseline CEC values (p = 0.02; Figure 3).

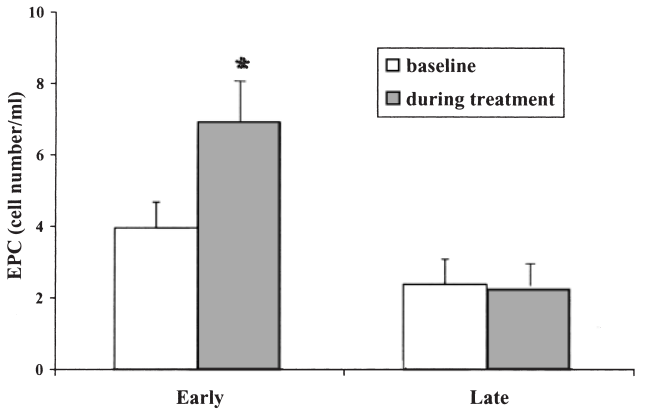

Figure 2. Levels of EPC at baseline and at the end of statin therapy in patients with SSc stratified according to disease duration. *p = 0.04 for SSc patients with recent-onset disease at baseline versus patients after 12 weeks of statin treatment. No difference was observed in patients with late SSc in EPC values at baseline versus the end of statin treatment.

Effects of simvastatin on serum cholesterol and cytokine levels. Treatment with simvastatin did not result in a decrease in total and LDL serum cholesterol levels in patients with SSc (Table 1).

Simvastatin-treated patients showed a significant reduction of the levels of soluble adhesion molecules (E-selectin, ICAM-1, and VCAM-1) and of endothelial-related cytokines (IL-6 and ET-1; Table 1). Interestingly, after discontinuation of therapy, E-selectin, VCAM-1, and ET-1 maintained statistically significant reduced levels in comparison with baseline values. In contrast, plasma levels of ICAM-1 and IL-6, cytokines known to be not of exclusively vascular origin, increased up to baseline concentrations. We did not evaluate the effect of statin discontinuation in the hypercholesterolemic group since therapy was not discontinued in that group.

Clinical effects. No changes in the Raynaud's phenomenon variables were observed during the study period. One patient developed new digital ulcers during statin treatment.

DISCUSSION

We evaluated the potential usefulness of statins in modulating endothelial activation and in increasing endothelial remodeling and repair in a small group of patients with SSc. This was done by measuring, before and after a short treat-

Table 1. Changes in inflammatory cytokines and biochemical markers of endothelial function in SSc patients undergoing simvastatin treatment. Data are expressed as mean ± SE.

	Baseline	During Treatment	After Discontinuation
Total cholesterol, mg/dl	154 ± 40	148 ± 41 (NS)	139 ± 28 (NS)
LDL cholesterol, mg/dl	128 ± 24	114 ± 35 (NS)	113 ± 41 (NS)
sE-selectin, ng/ml	52.30 ± 20.1	38.1 ± 10.2 (p = 0.0002)	46.1 ± 18.6 (p = 0.01)
sICAM-1, ng/ml	403 ± 128.2	298 ± 125 (p = 0.0008)	345 ± 156 (NS)
sVCAM-1, ng/ml	604 ± 220.4	408 ± 125 (p = 0.001)	502 ± 113 (p = 0.005)
IL-6, ng/ml	42 ± 10.3	25.7 ± 7.5 (p = 0.04)	35 ± 9.1 (NS)
Endothelin 1, pg/ml	4.88 ± 2.1	2.90 ± 1.8 (p = 0.03)	3.81 ± 1.9 (p = 0.05)

NS: nonsignificant.

ment course with simvastatin, the number of CEC and the levels of some cytokines, which are considered markers of endothelial damage and activation, respectively, and the number of EPC, which are commonly believed to be indicative of an attempt to repair the damaged endothelial surface.

On the whole, our study shows that in SSc, statins can be rather effective in controlling and limiting the endothelial activation and damage, but fail to significantly increase the mobilization of EPC from the bone marrow and the consequent vascular homing and repair.

Statins have been shown to possess multiple actions capable of modulating endothelial activation and reducing endothelial damage both in ischemic conditions and in inflammatory vascular disorders[15]. Besides the beneficial effects on vascular damage related to lowering of total cholesterol and oxidized LDL[16], statins are able to correct impaired nitric oxide bioavailability in atherosclerotic vessels by increasing endothelial nitric oxide synthase (eNOS), production, and activity[17,18]. In addition, statins are effective in reducing the expression of adhesion molecules and the production of other proinflammatory cytokines from the activated endothelium[19-22]. These actions can consequently modulate homing and migration of inflammatory cells through the vessel wall, which certainly play an important role in atherosclerotic plaque formation and rupture, and in the pathological process of a number of inflammatory vascular disorders[23,24].

In recent years, EPC have been shown to play an important role in vascular repair and the angiogenic process of tissues damaged by ischemia, providing new possibilities for local vascular repair by means of cell therapy in patients with peripheral or coronary artery disease[25-27]. There is also growing evidence that statins are capable of inducing mobilization and differentiation of EPC from the bone marrow, and consequently accelerating vasculogenesis and revascularization in damaged tissues[5-7,25-29]. This suggests a possible beneficial use of these drugs in the acute phase of diseases causing ischemic injuries[7,29,30].

Since SSc, at least in its first phase, can be considered an inflammatory disease involving the microvasculature of the skin and other organs and systems, with consequent ischemic damage[1], it may be postulated that statins could be useful in the treatment of this disorder by limiting the endothelial damage and also promoting repair. Kuwana, *et al* recently reported, from an open-label short-term therapeutic trial in a small group of patients with both diffuse and limited SSc, that atorvastatin was able to induce a significant increase of circulating EPC[8]. However, this effect is certainly lower than that observed in normal subjects and seems to be transient, at least in some of the studied patients. Moreover, the mobilized EPC appeared to be functionally defective. These results are not completely confirmed in our study, where the number of EPC was not significantly increased under treatment with simvastatin. It is notable that in our results, mobilization of EPC appeared to be significantly greater only in patients with early disease, compared to those with late disease. This result was not found in the previous report[8], where no differences were reported in the amount of EPC according to the disease duration. The different results we observed could be partially ascribed to the different statins used and to a different selection of patients for study (in our study, only those with a limited variant of SSc).

It is worth noting that different methods are used to measure circulating endothelial cells, and none is standardized, so it is difficult to compare one method to the others and results may conflict. Actually the values obtained using different methods to measure numbers of EPC could be totally different, and so not easily comparable. Moreover, this matter is developing with the expansion of knowledge in

this field. In most studies to date, EPC are defined and enumerated through flow cytometry by the expression of CD34, CD133, or VEGFR-2. That we found values of EPC in our previous studies that were quite different from those in the present investigation reflects that we characterized the EPC by the presence of CD133 only, and not more specifically by the presence of all these markers[4]. Since all these molecules are also expressed on hematopoietic stem/progenitor cell populations, the hematopoietic contamination of EPC is an expected phenomenon. However, the method we used here is the one used most frequently in recent studies and although it is not validated, it gives reliable results in different disease groups[3,31-33].

The fact that EPC mobilization from bone marrow is always reduced, compared to controls, confirms the hypothesis that impaired endothelial repair may also contribute to the pathogenesis and progress of the scleroderma vasculopathy. Multiple mechanisms may be postulated to explain the repair failure by EPC in SSc. First, the continuous request from the damaged peripheral endothelia may exhaust the bone marrow reservoir of endothelial-directed stem cells, in a manner similar to that shown in patients carrying multiple risk factors for cardiovascular disease[34]. Second, scleroderma-related damage of the microvascular network in the bone marrow may compromise the mechanisms that promote local mobilization of endothelial cell precursors, making the local stimulating actions of different cytokines, chemokines, and drugs ineffective. Whatever the mechanism of EPC failure is, our study confirms the previous findings about the presence in SSc of a complex impairment of the bone marrow microenvironment that is responsible for the well known numerical and functional defects of EPC in scleroderma[2,3]. Specific studies on bone marrow, not undertaken here, are undoubtedly needed to confirm the capacity of statins to mobilize EPC to repair peripheral endothelial damage, at least in the early phase of disease.

Our data confirm that statins are able to significantly reduce the different markers of endothelial damage and activation in patients with SSc, similarly to what has been observed in other vascular disorders[21-24]. In our study, the simvastatin preserved peripheral endothelial function in the absence of any lipid-lowering effects. This result is not surprising; different investigators have shown substantial evidence that among the pleiotropic effects of statins, the anti-inflammatory properties might represent one of the most important actions of lowering lipid levels[13].

Therefore, to ascribe the mild clinical improvement observed previously only to the effect of statins on partially ineffective EPC recruitment remains speculative, even considering that this beneficial effect is of a minor extent and is essentially limited to the early phase of the disease. In contrast, statins could predominantly act by downregulating endothelial activation and limiting subsequent damage at the peripheral vascular level. To confirm our preliminary data, a double-blind controlled study in a larger group of patients with early limited disease, where the microvascular process is recognized to play a key role, could represent a more adequate experimental design.

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