Transcriptional Regulation of Bim by FOXO3a and Akt Mediates Scleroderma Serum–Induced Apoptosis in Endothelial Progenitor Cells

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Background—Endothelial progenitor cells (EPCs) contribute to vascular regeneration/repair and thus may protect against scleroderma vasculopathy. We aimed to determine whether circulating EPCs were reduced in scleroderma, whether scleroderma sera could induce EPC apoptosis, and, if so, what the underlying apoptotic signaling pathway was.

Methods and Results—Circulating EPC levels were quantified in 54 patients with scleroderma and 18 healthy control subjects by colony-forming unit assay and flow cytometry, which revealed markedly decreased EPC levels in scleroderma patients relative to healthy subjects. Substantial apoptosis was detected in EPCs after culturing in the presence of scleroderma sera compared with normal sera. Intriguingly, depletion of the IgG fraction from scleroderma sera completely abolished the apoptotic effects. Furthermore, scleroderma sera inhibited the activation/phosphorylation of Akt, which in turn suppressed the phosphorylation and degradation of forkhead transcription factor FKHRL1 (FOXO3a), resulting in the upregulation of apoptotic protein Bim. siRNA-mediated FOXO3a and Bim knockdown substantially reduced scleroderma serum–induced EPC apoptosis. Importantly, Bim expression and baseline apoptosis were increased in EPCs freshly isolated from scleroderma patients relative to that obtained from healthy subjects.

Conclusion—Scleroderma serum–induced EPC apoptosis is mediated chiefly by the Akt-FOXO3a-Bim pathway, which may account, at least in part, for the decreased circulating EPC levels in scleroderma patients. (Circulation. 2008;118:2156-2165.)

Key Words: angiogenesis ■ antibodies ■ apoptosis ■ endothelial progenitor cells ■ scleroderma

Systemic sclerosis (SSc), or scleroderma, is a chronic, multisystem connective tissue disease affecting the skin and various internal organs. The disease is characterized by the triad of vascular damage, fibrosis, and autoimmunity. Although the relationship between these 3 pathological features is not fully understood, the observation that Raynaud’s phenomenon and other disturbances in the peripheral vascular system precede the onset of fibrosis in SSc raises the possibility that fibrosis in SSc may represent a default pathway resulting from vascular failure. Hence, understanding the pathobiology of SSc vascular disease is the key in dissecting the disease pathogenesis and developing novel therapeutic strategies.

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The circulatory damage in SSc affects primarily the microvasculature and small and medium-sized arteries, resulting in chronic underperfusion and ischemia in affected skin and internal organs. These changes were expected to trigger an extensive compensatory angiogenic response. Numerous studies, however, have indicated that angiogenesis, a process mediated chiefly by endothelial progenitor cells (EPCs) derived from the bone marrow, is impaired in SSc despite elevated levels of vascular endothelial growth factor (VEGF), a potent angiogenic factor, in the peripheral blood. Any defect in the EPC supply within and mobilization from the bone marrow or excessive destruction of EPCs on their mobilization by immune system could potentially impede vascular regeneration/repair. We recently demonstrated that transplantation of SSc skin into severe combined immunodeficient (SCID) mice elicited robust angiogenesis, an effect that was significantly stronger than normal skin grafting, and that the neovascular cells were derived almost exclusively from the recipients, perhaps originating from the bone marrow. These data indicate that SSc skin is capable of releasing factors that mobilize EPCs from the bone marrow and narrow the possibilities for angiogenesis/vascular repair defects in SSc down to EPC depletion within the bone marrow and/or EPC destruction in the circulation.

FOXO3a is a member of FOXO subfamily of forkhead transcription factors, which regulate cell survival and apopto-
sis.\textsuperscript{6} In the presence of survival factors, Akt, the survival kinase, is phosphorylated and activated, which in turn phosphorylates FOXO3a, leading to association with 14-3-3 proteins, nuclear exclusion, and retention and degradation of FOXO3a in the cytoplasm. Conversely, the presence of apoptosis factors inhibits Akt phosphorylation, which leads to FOXO3a dephosphorylation, nuclear translocation, and activation of FOXO target genes, including the proapoptotic genes Bim and Puma, resulting in cell apoptosis.\textsuperscript{6}

Contradictory results have been reported regarding the levels of circulating EPCs in SSC.\textsuperscript{7,8} Although SSC sera are incapable of inducing mature endothelial cell (EC) apoptosis, little is known regarding the effects of SSC sera on EPCs. In this study, we examined the levels of circulating EPCs in 5 cohorts of patients: (1) early-stage diffuse cutaneous SSC (dcSSC), (2) intermediate/late-stage dcSSC, (3) early-stage limited cutaneous SSC (lcSSC), (4) intermediate/late-stage lcSSC, and (5) healthy control subjects. We found a significant reduction in circulating EPC levels in all disease phenotypes compared with healthy subjects. We provide evidence that SSC, but not normal, sera induce EPC apoptosis via the Akt-FOXO3a-Bim pathway, which may account, at least in part, for the reduction in circulating EPCs in SSC.

Methods

The following provides a description for each of the methods used in the study. Detailed description is found in the online Data Supplement.

Subject Characteristics

Fifty-four patients (14 men, 40 women; age, 56.9±12.6 years; median disease duration, 8.5 years; range, 0.17 to 40 years) with SSC as defined by the American College of Rheumatology SSC classification criteria\textsuperscript{9} presenting to Duke Hospital for treatment were included in this study. Patients with overlap connective tissue syndromes, previous myocardial infarction, stroke, valvular or congenital heart disease, congestive heart failure, diabetes mellitus, hypertrophic cardiomyopathy, dyslipidemia, history of smoking, or history of arterial hypertension before the diagnosis of SSC were excluded. Patients were classified into 4 groups (Table I of the online Data Supplement) based on SSC disease type (dcSSC or lcSSC) and duration (from the onset of the first non-Raynaud’s symptom of scleroderma). They are as follows: (1) 12 patients with early-stage lcSSC (sclerosis confined to areas distal to the elbows or knees and above the clavicles; disease duration <5 years); (2) 13 patients with intermediate/late-stage lcSSC (disease duration ≥5 years); (3) 10 patients with early-stage dcSSC (sclerosis extending proximal to the elbows or knees with or without truncal involvement; disease duration <3 years); and (4) 19 patients with intermediate/late-stage dcSSC (disease duration ≥3 years). Disease stages were defined as suggested by LeRoy et al.\textsuperscript{10} Eighteen healthy subjects undergoing diagnostic cardiac catheterization at Duke University Medical Center showing no evidence of coronary artery diseases were used as control subjects in the study.

Peripheral Blood Collection

After informed consent was obtained from the subjects, an average of 30 mL peripheral blood was collected from each patient.

Colony-Forming Unit Assay

Colony-forming assay was performed as described by Hill et al.\textsuperscript{11}

Flow Cytometry Analysis

Mononuclear cells were isolated by density centrifugation as described in the online Data Supplement.

Results

Patient Characteristics

The patient characteristics are presented in supplemental Table I. The mean age of the lcSSC cohort tended to be higher than that of the dcSSC cohort. More than 60% of subjects were female in all SSC patient groups. Factors that may affect EPCs,\textsuperscript{11} including pulmonary hypertension, statin therapy, and corticosteroid therapy, were recorded. These factors were taken into account in the analyses and interpretation of the EPC data.

Reduced Circulating EPCs in SSC Patients

We first used colony-forming unit assay,\textsuperscript{11} an established method at the time when this study was conducted, to measure circulating EPCs. We now know that EPCs detected by this method correspond to early EPCs.\textsuperscript{12} Nevertheless, we were able to determine the endothelial nature of the cells within the colony. Indeed, these cells stained positive with multiple endothelium-specific markers, in particular von...
Willebrand factor and VEGF receptor 2 (VEGFR2; Figure 1). The number of circulating early EPCs was dramatically reduced in the lcSSc group ($n=26$) and dcSSc group ($n=29$) compared with the healthy control group ($1.1 \pm 0.45$ for lcSSc and $1.5 \pm 0.46$ for dcSSc versus $26.9 \pm 2.2$ for the control group; $P=0.0001$) when both early-stage and intermediate/late-stage diseases were analyzed as a whole (Figure 1). The difference between the lcSSc and dcSSc cohorts was not statistically significant ($P=0.863$). It was previously shown that the EPC level was higher in early-stage than in late-stage SSC patients. To further study the changes in early EPC levels relative to SSC disease duration, we stratified and analyzed our data obtained in the substantial number of patients in each of the subgroups. Interestingly, there was no significant difference in the levels of circulating early EPCs in early versus intermediate/late lcSSc ($P=0.25$) or early versus intermediate/late dcSSc ($P=0.88$). All SSC subgroups had lower early EPC levels than healthy subjects (Figure 1G). We also analyzed the complicating effects of pulmonary arterial hypertension and statin and corticosteroid therapies on circulating early EPC levels were observed by multinomial logistic regression analysis.

Because there is controversy regarding the definition and enumeration of circulating EPCs, in particular with the use of colony-forming assay, to confirm the above findings and to provide a robust analysis of EPCs that may be phenotypically different from the early EPCs identified by colony forming assay, flow cytometry was used to determine the number of EPCs coexpressing CD133/CD34, CD133/VEGFR2, or CD133/CD34/VEGFR2 in the peripheral blood as shown in supplemental Figure 1. Circulating CD133+/CD34+, CD133+/VEGFR2+, and CD133+/CD34+/VEGFR2+ cell levels were significantly lower in the lcSSc and dcSSc patients compared with the healthy control subjects (Figure 2). There was no significant difference between the lcSSc and dcSSc cohorts. The differences between early and intermediate/late lcSSc and between early and intermediate/late dcSSc were not statistically significant. Moreover, there were no significant differences for the various EPC phenotypes among the different SSC patient cohorts (data not shown). The presence or absence of pulmonary arterial hypertension, statin therapy, and corticosteroid therapy did not affect the levels of any of the EPC phenotypes (data not shown). These data indicate that decreased circulating EPCs may be an integral part of the SSC vascular disease that often precedes the onset of fibrosis.
involved 2 steps: heat inactivation at 56°C for 30 minutes and IgG depletion (Figure 3B). The IgG depletion completely abolished the apoptosis-inducing effect of SSc sera, whereas no difference was observed with normal sera with and without IgG depletion (Figure 3B). As shown in Figure 3B, heat inactivation alone did not change the effects of SSc and normal sera on EPC apoptosis. These observations exclude the involvement of complements in SSc serum–induced EPC apoptosis and indicate that EPCs may be destroyed on their mobilization from the bone marrow by heat-resistant factors, including autoantibodies, present in the IgG fraction of SSc sera. Thus implicating a potential role of EPC reduction in the pathogenesis of SSc, a notion that is consistent with the prevailing view in the field.13

Induction of Apoptosis in EPCs by SSc Sera
To determine whether excessive EPC destruction by autoantibodies present in the sera contributed to the reduction of circulating EPCs in SSc patients, we examined the apoptotic effects of SSc sera on EPCs in vitro. CD133+ EPCs isolated from human UCB or bone marrow were cultured in the presence of each serum sample from lcSSc patients (n=5), dcSSc patients (n=5), and healthy control subjects (n=5) for 48 hours. At a concentration of 10%, all lcSSc and dcSSc samples showed rather consistent proapoptotic effects, whereas serum samples from healthy subjects had no effect on EPC apoptosis, relative to fetal bovine serum (FBS). Hence, in the following experiments, pooled sera from 5 lcSSc and 5 dcSSc samples (SSc sera) and pooled sera from 5 healthy subjects (normal sera) were used. SSc sera dose dependently induced UCB-derived EPC apoptosis as detected by DNA fragmentation, whereas normal sera had no apoptotic effects (Figure 3A). A similar degree of apoptosis was observed in bone marrow–derived EPCs when treated with SSc sera but not with normal sera (data not shown). Thus, in the following experiments, we exclusively used EPCs isolated from UCB because of its ready availability to us. The apoptosis findings in UCB-derived EPCs were further confirmed by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) and caspase 3/7 assay, confirmed by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) and caspase 3/7 assay, as defined by CD133+/CD34− cells (Ⅴ), CD133+/VEGFR2+ cells (Ⅲ), or CD133+/CD34−/VEGFR2− (■) cells, are substantially decreased in lcSSc and dcSSc patients vs the control group. There is no significant difference for various EPC phenotypes between the 2 patient groups. Significance was determined by 1-way ANOVA followed by Dunnett’s test to compare the level of EPCs in different disease groups and the control group. *P<0.05, **P<0.0001. PBMCs indicates peripheral blood mononuclear cells.

Figure 2. Decreased EPCs in SSc patients by fluorescent-activated cell sorter analysis. The levels of circulating EPCs, as defined by CD133+/CD34− cells (Ⅴ), CD133+/VEGFR2+ cells (Ⅲ), or CD133+/CD34−/VEGFR2− (■) cells, are substantially decreased in lcSSc and dcSSc patients vs the control group. There is no significant difference for various EPC phenotypes between the 2 patient groups. Significance was determined by 1-way ANOVA followed by Dunnett’s test to compare the level of EPCs in different disease groups and the control group. *P<0.05, **P<0.0001. PBMCs indicates peripheral blood mononuclear cells.

EPCs Are More Susceptible Than Mature ECs to SSc Sera
Anti-EC antibodies, found in the serum of SSc patients, are known to induce EC apoptosis both in vitro and in vivo.2 Hence, it has been assumed that increased endothelial apoptosis is the earliest microvascular abnormality in SSc that precedes fibrotic changes. Yet, the deficiency of EPC-mediated repair and its contribution to SSc vascular lesion formation relative to endothelial injury have not been studied before. To address this important question, we treated human microvascular ECs with sera obtained from lcSSc and dcSSc patients and compared them with EPCs treated in the same fashion. As expected, SSc sera induced apoptosis in a significant number of human microvascular ECs compared with normal sera or FBS. Intriguingly, ECs were 5.5-fold less susceptible than UCB-derived EPCs to the toxic factors present in the SSc sera (Figure 3B). These data suggest that the defective repair, mediated at least in part by serum-induced circulating EPC apoptosis, might be more important than vascular damage in SSc vascular lesion formation.

SSc Sera Regulate Akt-FOXO3a-Bim Pathway in EPCs
FOXO transcription factors are phosphorylated by Akt, which leads to cytoplasmic retention and impairment of FOXO nuclear transcriptional activity.6 FOXO3a has been shown to mediate apoptosis in glioma,14 and FOXO4 was involved in H2O2–induced apoptosis in EPCs isolated from peripheral blood through the induction of the proapoptotic protein Bim.15 To determine which FOXO proteins in UCB-derived EPCs were most affected by SSc sera, we performed Western blotting for FOXO1, FOXO3a, and FOXO4 in cells treated with SSc and normal sera. Endogenous levels of all 3 FOXO proteins were detected in cells cultured in FBS and normal sera. FOXO3a expression was markedly increased in cells treated with SSc sera, whereas the expression of FOXO1 and FOXO4 was essentially unchanged (Figure 4), pointing to a potential role of FOXO3a in regulating EPC apoptosis. Hence, in the following experiments, we focused on FOXO3a.

We examined whether SSc sera with and without IgG depletion affected the phosphorylation and expression of Akt and FOXO3a. As shown in Figure 5, Akt and FOXO3a were constitutively phosphorylated in EPCs treated with normal or IgG-depleted SSc sera. Treatment of these cells with SSc sera resulted in dephosphorylation of both Akt and FOXO3a. SSc

protein G binding. To exclude the possibility that inactivation of complements that are sensitive to heat treatment might account for the lack of apoptosis-inducing effects observed with IgG-depleted SSc sera, we treated EPCs with SSc and normal sera with heat inactivation but without IgG depletion. As shown in Figure 3B, heat inactivation alone did not change the effects of SSc and normal sera on EPC apoptosis. Hence, it has been assumed that increased endothelial apoptosis is the earliest microvascular abnormality in SSc that precedes fibrotic changes. Yet, the deficiency of EPC-mediated repair and its contribution to SSc vascular lesion formation relative to endothelial injury have not been studied before. To address this important question, we treated human microvascular ECs with sera obtained from lcSSc and dcSSc patients and compared them with EPCs treated in the same fashion. As expected, SSc sera induced apoptosis in a significant number of human microvascular ECs compared with normal sera or FBS. Intriguingly, ECs were 5.5-fold less susceptible than UCB-derived EPCs to the toxic factors present in the SSc sera (Figure 3B). These data suggest that the defective repair, mediated at least in part by serum-induced circulating EPC apoptosis, might be more important than vascular damage in SSc vascular lesion formation.
serum treatment also increased the level of total FOXO3a but did not affect the expression level of total Akt in EPCs. In contrast, normal sera with and without IgG depletion, IgG-depleted SSc sera, and FBS had no effect on total Akt and FOXO3a expression (Figure 5).

We then examined the factors downstream of Akt-FOXO3a signaling, namely Bim and Puma. The expression of Bim protein was significantly increased in EPCs treated with SSc sera compared with normal sera. The inducing effect of SSc sera on Bim expression was almost completely abolished by IgG depletion. A similar but much less dramatic effect was observed in Puma expression in response to SSc and normal serum treatment (Figure 5). These changes were further confirmed by real-time reverse-transcriptase polymerase chain reaction. The mRNA levels for Bim and, to a much lesser degree, for Puma were higher in EPCs treated with SSc sera than that observed in the presence of normal sera or IgG-depleted SSc sera (Figure 6). Importantly, the level of Akt dephosphorylation correlated with that of FOXO3a dephosphorylation and the induction of Bim expression. These data indicate that factors present in SSc sera, particularly in the IgG fraction, may inactivate Akt, which, in turn, dephosphorylates and activates FOXO3a, resulting in the induction of proapoptotic proteins Bim and, to a lesser degree, Puma. Collectively, these findings suggest that SSc serum–induced EPC apoptosis might be mediated by the Akt-FOXO3a-Bim pathway.

FOXO3a Silencing Reduces SSc Serum–Induced Bim Expression and Apoptosis in EPCs

To confirm the role of the Akt-FOXO3a-Bim pathway in SSc serum–induced EPC apoptosis, we performed gene-silencing experiments. EPCs were transfected with a mixture of 3 sets of siRNA duplexes specific for FOXO3a or a scrambled siRNA control (Figure 7A and 7B). The cells were then cultured in the presence of SSc sera, normal sera, or FBS.

Figure 3. SSc sera induce apoptosis in EPCs. SSc sera dose dependently induce UCB-derived EPCs to undergo apoptosis, as determined by DNA fragmentation ELISA assay (A). The apoptosis effect of 10% SSc sera vs normal sera or FBS is confirmed by caspase 3/7 assay; remarkably, depletion of IgG from SSc sera, but not heat inactivation, abolishes the apoptosis-inducing effect of SSc sera (B). Furthermore, EPCs are 5.5-fold more sensitive to SSc serum–induced apoptosis than human microvascular ECs (HMVEC; B). TUNEL labeling also shows substantial EPC apoptosis in the presence of 10% SSc sera: nuclear staining by Hoechst 3342 (C, F), TUNEL staining (D, G), and overlay (E, H). Significance was determined by 1-way ANOVA followed by Dunnett’s test to compare the caspase activity in different conditions and the normal serum treatment. *P<0.05, **P<0.0001.

Figure 4. SSc sera induce FOXO3a expression in EPCs. Endogenous levels of FOXO1, FOXO3a, and FOXO4 expression are detected in UCB-derived EPCs. SSc sera markedly induce the expression of FOXO3a, but not FOXO1 and FOXO4.
FOXO3a siRNA markedly reduced endogenous FOXO3a expression (data not shown). Furthermore, SSc sera failed to induce the phosphorylation of FOXO3a and Bim expression in FOXO3a siRNA–transfected cells compared with scrambled siRNA transfection (Figure 7C and 7D). In contrast, the effect of FOXO3a knockdown on the endogenous expression and SSc serum–induced induction of Puma was, if anything, trivial (data not shown). We then determined whether the reduction in FOXO3a and Bim levels resulting from FOXO3a siRNA treatment could inhibit SSc serum–induced apoptosis in EPCs. We repeated the siRNA knockdown experiments and measured DNA fragmentation using ELISA. As expected, apoptosis in FOXO3a siRNA–transfected EPCs in the presence of SSc sera was suppressed to a level similar to that observed in cells treated with FBS and normal sera (Figure 7E). These data indicate that FOXO3a is crucial in the activation of Bim expression and the induction of EPC apoptosis.

**Bim Silencing Reduces SSc Serum–Induced EPC Apoptosis**

To further confirm that Bim serves as the important factor downstream of Akt-FOXO3a signaling in mediating SSc serum–induced EPC apoptosis, CD133+/H11001 EPCs were transfected with Bim-specific or control scrambled siRNA. As shown in Figure 8A, transfection with Bim siRNA decreased Bim mRNA expression by 65% compared with control siRNA. The level of apoptosis in Bim siRNA–transfected EPCs was approximately one third of that seen in scrambled siRNA-transfected EPCs in the presence of SSc sera (Figure 8B). Thus, Bim siRNA transfection in EPCs resulted in a similar degree (about two thirds) of inhibition of Bim expression and apoptosis. These findings highlight the critical role of Bim in mediating SSc serum–induced EPC apoptosis. Our data, however, cannot exclude the potential minor contributions by other proapoptotic pathways shown to be regulated by FOXO3a, including jun N-terminal kinase activation and Flice inhibitory protein downregulation, to EPC apoptosis induced by SSc sera.16

**Increased Bim Expression and Baseline Apoptosis in SSc Circulating EPCs**

Because treatment with SSc sera induced the dephosphorylation of Akt and activation of FOXO3a, resulting in the upregulation of Bim expression and apoptosis in UCB-derived EPCs, we reasoned that circulating EPCs derived from SSc patients should have increased Bim expression and baseline apoptosis as a result of constant exposure to SSc sera.16
sora. To test this possibility and to establish the missing link between the activation of the Akt-FOXO3a-Bim pathway in vitro and the decreased EPC levels in SSc patients in vivo, we measured the expression levels of Bim expression in CD133\(^+\)/H11001 EPCs isolated from dcSSc and lcSSc patients relative to healthy subjects using real-time reverse-transcriptase polymerase chain reaction. Because of the low frequency of CD133\(^+\)/H11001 EPCs in the peripheral blood, we pooled the cells isolated from the peripheral blood of 9 dcSSc patients, 9 lcSSc patients, and 9 healthy subjects into 3 tubes per group. As shown in Figure 8C, Bim expression was significantly and substantially increased in EPCs obtained from dcSSc and lcSSc patients compared with those isolated from healthy subjects. Remarkably, freshly isolated CD133\(^+\) EPCs from SSc patients showed increased baseline apoptosis when cultured in endothelial growth medium containing 10% FBS for 18 hours (Figure 8D). These data indicate that data generated from our in vitro study are highly relevant to the pathophysiological changes in vivo and support the notion that the Akt-FOXO3a-Bim axis plays a central role in the increased EPC apoptosis in SSc patients.

**Discussion**

Several lines of evidence indicate that progressive vascular disorder is a primary event in the pathogenesis of SSc.\(^2\) Indeed, the observation that Raynaud’s phenomenon precedes the diagnosis of SSc by months or years has prompted the speculation that fibrosis in SSc may represent a default pathway resulting from vascular failure.\(^3\) Previous studies related to the pathogenesis of SSc vascular disease have focused almost exclusively on vascular injury. Specifically, endothelial apoptosis is thought to be an initiating event in SSc vascular lesion formation.\(^17\) Since the identification of circulating EPCs in 1997,\(^18\) the field of vascular biology has experienced a significant paradigm shift with the introduction of EPC-induced vascular repair. Indeed, the availability of progenitor cells is closely linked to the initiation and development of several vascular disorders, including atherosclerosis.\(^19\) Thus, characterizing the vascular repair aspect, namely EPCs, in SSc would likely shed light on the understanding of the pathogenesis of SSc vascular lesions. In this report, we provide data demonstrating substantial depletion of circulating EPCs in SSc patients, regardless of the disease subtypes and the different methodologies used to measure EPCs.

Conflicting data have been reported regarding the levels of EPCs in SSc patients. Kuwana et al\(^7\) demonstrated a reduced number of CD34\(^+\)/CD133\(^+\)/VEGFR2\(^+\) EPCs with impaired function in SSc patients. Their findings are consistent with our observations. Very recently, Avouac et al\(^20\) however, showed that the level of the same type of EPCs was increased in SSc patients relative to healthy subjects. Although all these studies used flow cytometry to detect EPCs, different gating may have contributed to the discrepancy. Furthermore, 2 different types of EPCs appear to exist: early and late EPCs,
according to their time-dependent appearance when they are isolated with cell culture–based methods.12 The 2 types of EPCs have different morphologies, gene expression profiles, and survival behaviors. Both types of EPCs, however, contribute to neovasculogenesis in vivo; early EPCs secrete mainly angiogenic cytokines, whereas late EPCs supply a sufficient number of ECs.21 Interestingly, we demonstrated that early EPCs were markedly decreased in SSc patients. In contrast, Avouac et al20 found that the levels of late EPCs were not different between SSc patients and healthy subjects. The pathogenetic significance of the decreased early EPCs versus unchanged late EPCs remains to be determined in SSc.

The initial stages of SSc are generally not accessible for analysis in humans. Hence, we classified the dcSSc and lcSSc disease cohorts into early and intermediate/late stages, hoping that the early stage could reflect some of the pathobiological changes occurring in the initial stages of SSc. In contrast to the findings made by Del Papa et al8 showing that CD45+/CD133+ EPC levels were decreased in intermediate/late-stage but increased in early-stage SSc patients, we found that EPCs were substantially and consistently decreased in both early- and intermediate/late-stage SSc patients. These data, coupled with the observation that bone marrow precursors play an important role in inducing angiogenesis in SSc skin grafts in SCID mice5 and numerous reports supporting the critical role of EPCs in the initiation of other vascular diseases such as atherosclerosis, indicate that the lack of circulating EPCs may also be important in the initiation of SSc vascular lesion formation. The discrepancy between our data and those of Del Papa et al8 may be due to differences in EPC definition and measurements used in the 2 studies. Specifically, EPCs were defined as CD45+/CD133+ cells by Del Papa et al, whereas several EPC phenotypes, including CD133+/CD34+, CD133+/VEGFR2+, and CD133+/CD34+/VEGFR2+ cells and those determined by the colony-forming assay, were investigated in our study.

EC apoptosis induced by anti-EC antibodies is thought to be one of the earliest steps in the vascular pathology of SSc.22 The integrity of the endothelium is ensured by effective EC repair under steady-state conditions, which is mediated by the proliferation of adjacent ECs and, more important, by circulating EPCs. It has been documented that, in the setting of

![Figure 8. Bim is critical in regulating EPC apoptosis. Bim siRNA (siBim), but not control siRNA (siControl), transfection reduces Bim expression (A) and inhibits SSc serum–induced EPC apoptosis (B). CD133+ EPCs isolated from lcSSc and dcSSc patients show increased endogenous Bim expression (C) and enhanced baseline apoptosis (D) relative to those obtained from healthy subjects. Values were obtained from experiments in triplicate and repeated at least twice. Significance was tested by 1-way ANOVA followed by Dunnett’s test or unpaired Student t test to compare the levels of Bim expression and apoptosis in SSc patients and control subjects. *P < 0.05, **P < 0.0001.](http://circ.ahajournals.org/)
atherosclerosis, disease risk factors diminish the supply of EPCs needed to maintain the homeostasis of the cardiovascular system, tilting the balance of vascular injury and repair in favor of injury and atherosclerosis progress. To determine whether autoimmunity would cause EPCs to undergo apoptosis, resulting in impaired repair capacity in SSc, we examined whether EPCs were sensitive to autoantibody-induced cell death. Indeed, using multiple assays, we demonstrated, for the first time, substantial EPC apoptosis when the cells were treated with sera from patients with SSc. Furthermore, depletion of IgG from SSc sera abolished EPC apoptosis, suggesting the involvement of autoantibodies in this process. These findings indicate that SSc serum–induced EPC apoptosis may serve as a major putative mechanism for the decreased circulating EPC levels in SSc. In addition to inducing apoptosis, SSc sera may exert inhibitory effects on EPC proliferation. Given the greater sensitivity of EPCs than ECs to SSc sera, it seems that defective repair may be more important than vascular damage in SSc vascular lesion formation.

Little is known about the molecular signaling pathway underlying EPC apoptosis, particularly in response to SSc serum exposure. It has been shown that FOXO proteins are important regulators for the fate of hematopoietic stem cells. The Akt-FOXO3a-Bim axis has been implicated in glioma cell apoptosis. Furthermore, FOXO4-dependent expression of Bim has been shown to mediate reactive oxygen species–induced EPC apoptosis. Studies in other cell systems showed that FOXO3a could directly modulate Bim expression and induce apoptosis. We surveyed FOXO1, FOXO3a, and FOXO4 and found that, although all 3 FOXO proteins were present in UCB-derived EPCs, only FOXO3a was affected by SSc sera. Consistent with previous observations showing the cause-and-effect relationship between Akt and FOXO3a, we showed that both Akt and FOXO3a were dephosphorylated in EPCs in the presence of SSc sera. Furthermore, SSc serum treatment upregulated the expression level of FOXO3a and Bim and induced EPC apoptosis. Intriguingly, IgG depletion from SSc sera abolished these effects. These data indicate that the Akt-FOXO3a-Bim axis plays an important role in mediating SSc serum–induced EPC apoptosis.

To further establish the central role of FOXO3a and Bim in mediating SSc serum–induced EPC apoptosis, we performed siRNA knockdown experiments. As expected, transection of EPCs with FOXO3a-specific siRNA silenced FOXO3a expression and abolished SSc serum–induced upregulation of Bim. Furthermore, the silencing of either FOXO3a or Bim substantially reduced SSc serum–induced EPC apoptosis. Importantly, EPCs isolated from SSc patients display increased Bim expression and baseline apoptosis. Taken together, our data support the notion that SSc serum–induced EPC apoptosis via the Akt-FOXO3a-Bim signaling pathway may be responsible for reduced EPC levels in SSc.

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Disclosures
None.

References
The origin of systemic sclerosis is still not completely understood but appears to be autoimmune. Immune activation targets mature endothelial cells, resulting in vascular integrity breakdown and ensuing fibrosis. Endothelial progenitor cells (EPCs) are bone marrow–derived nonleukocyte cells that participate in vascular repair and homeostasis. It has been shown that injury of endothelial cells not only induces a cascade of proinflammatory events, contributing to vascular lesion formation, but also stimulates the mobilization of EPCs from the bone marrow, mediating vascular repair. Hence, the availability of circulating EPCs plays a critical role in maintaining the integrity and functional activity of the endothelial monolayer and in vasculogenesis. Several lines of evidence indicate that increased demand for vascular repair in the context of repeated injury could exhaust the supply of EPCs in the bone marrow, interrupting the balance between vascular repair and injury. In this report, we provide evidence showing, for the first time, that the factors in the peripheral blood of systemic sclerosis patients that cause endothelial cell injury also may damage EPCs. Moreover, probably owing to the lack of protective mechanisms in these immature cells, EPCs are more sensitive to the toxic factors than endothelial cells, implicating excessive EPC destruction in the pathogenesis of systemic sclerosis. Importantly, we have identified an Akt-FOXO3a-Bim pathway to mediate EPC apoptosis. Although much work needs to be done to determine the exact factors in the systemic sclerosis serum causing EPC apoptosis, targeting the Akt-FOXO3a-Bim pathway may be considered a venue for future therapies.
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