Increased Circulating Concentrations of the Counteradhesive Proteins SPARC and Thrombospondin-1 in Systemic Sclerosis (Scleroderma). Relationship to Platelet and Endothelial Cell Activation

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ABSTRACT. ***Objective.*** To determine whether circulating concentrations of the counteradhesive proteins SPARC (secreted protein acidic and rich in cysteine) and thrombospondin-1 (TSP-1) are elevated in scleroderma (systemic sclerosis, SSc). The relationship of these counteradhesive proteins to measures of platelet and endothelial cell activation was examined.

Methods. Plasma from 45 patients with SSc (26 limited form, 19 diffuse) and 22 age and sex matched controls was assayed for SPARC, TSP-1, ß-thromboglobulin (ßTG), and platelet factor 4 (PF4), 2 distinct platelet α-granule products, and soluble E-selectin, a marker of endothelial cell activation.

Results. The mean (± SE) SPARC concentration was greater in patients with limited SSc (124.0 ± 9.6 ng/ml) compared to controls (66.8 ± 8.0 ng/ml) ($p = 0.0005$), whereas in patients with diffuse SSc (74.1 ± 7.9 ng/ml) it was not. Elevated SPARC concentrations in the limited SSc group could not be ascribed to either platelet or endothelial cell activation. TSP-1 concentrations were also increased in SSc patients ($n = 29$) compared to controls ($n = 11$) (2.98 ± 0.12 vs 2.4 ± 0.21 log transformed ng/ml; $p < 0.02$). Unlike SPARC, TSP-1 concentrations correlated with both ßTG ($r = 0.57$, $p = 0.0014$) and PF4 ($r = 0.41$, $p = 0.026$) levels, indicating that increased TSP-1 could, in part, be explained through elevated platelet α-granule release in SSc patients. Plasma levels of ßTG, PF4, and E-selectin were each similarly elevated ($p < 0.003$) in patients with both limited and diffuse SSc compared to controls.

Conclusion. That circulating SPARC and TSP-1 are elevated in patients with SSc raises the possibility that counteradhesive proteins, which regulate vascular organization and remodeling, might contribute to the pathogenesis of SSc vasculopathy. (J Rheumatol 2002;29:2565–70)

Key Indexing Terms:

SYSTEMIC SCLEROSIS | SPARC | THROMBOSPONDIN-1
PLATELET FACTOR 4 | ß-THROMBOGLOBULIN | E-SELECTIN

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Scleroderma (systemic sclerosis, SSc), a multisystem disorder of unknown etiology, is characterized by vascular dysfunction, autoantibody production, and eventual tissue fibrosis[1]. Early microvascular changes include gaps between endothelial cells, perivascular edema, recruitment of mononuclear and mast cells, and intravascular platelet aggregation[2,3]. Injury to the microvasculature appears to precede perivascular recruitment of inflammatory cells. What in turn triggers impaired fibroblast activity with resultant excess deposition of extracellular matrix proteins is unknown[2-5].

In clinically unaffected and histologically normal skin from patients with scleroderma, increased endothelial cell surface expression of endothelial leukocyte-adherence molecule type 1 (ELAM-1 or E-selectin) can be detected[4]. Interestingly, circulating concentrations of soluble E-selectin are not only elevated in patients with scleroderma

compared to healthy controls[6], but are specifically elevated in patients with early as compared to late stage scleroderma[5]. In addition, elevated levels of circulating platelet aggregates and selected products of platelet α-granule release are consistent with *in vivo* platelet activation in patients with scleroderma[3,7,8]. On the basis of such findings, it has been proposed that, in scleroderma, an as yet undefined stimulus provokes endothelial cell injury and dysfunction with a subsequent dysregulated inflammatory and fibrotic host response.

The counteradhesive proteins are a group of structurally dissimilar multidomain proteins expressed in multiple tissues, grouped together solely on a functional basis[9-11]. These proteins inhibit cell adhesion to the extracellular matrix (ECM) and induce focal adhesion disassembly[9-11]. Only recently has it been appreciated that the counteradhesive proteins can influence endothelial cell–cell interactions[12-14]. Their expression is evident in highly proliferating, rapid-turnover epithelial cells and in tissues undergoing remodeling or wound healing[10,11]. The 3 principal members of the counteradhesive protein family are SPARC (secreted protein acidic and rich in cysteine), also known as osteonectin, thrombospondin-1 (TSP-1), and tenascin[9]. Two counteradhesive proteins, SPARC and TSP-1, share features that are relevant to the pathogenesis of scleroderma. Both proteins are produced by endothelial cells and both reside within platelet α-granules that are released upon platelet activation[9-11].

Despite their potential relevance, only a few reports have addressed counteradhesive proteins in scleroderma. Increased tenascin has been detected in the skin[15] and bronchoalveolar lavage fluids[16] of patients with scleroderma. Tenascin expression in dermal fibroblasts from patients with scleroderma is not increased whereas it is elevated in their lung fibroblasts compared to fibroblasts from healthy controls[16,17]. In another study, fibroblasts cultured from patients with SSc displayed increased SPARC mRNA expression[18]. To our knowledge, TSP-1 has not been studied in patients with scleroderma. Using a case-control study design, we investigated if circulating levels of the counteradhesive proteins SPARC and TSP-1 are elevated among patients with scleroderma. A secondary goal was to evaluate the association of these counteradhesive proteins with ß-thromboglobulin (ßTG) and platelet factor-4 (PF4), measures of *in vivo* platelet activation, and with soluble E-selectin, a measure of endothelial cell activation, in the circulation of patients with scleroderma.

MATERIALS AND METHODS

Patients. Forty-five patients diagnosed with scleroderma according to American College of Rheumatology criteria[19] were randomly selected from the Johns Hopkins and University of Maryland Scleroderma Center cohort. Each case patient was examined; clinical data were collected by 2 investigators (FMW and BW). In addition, 22 healthy volunteer controls were studied. Among the scleroderma patients, 26 were classified as having limited and 19 diffuse cutaneous disease[20]. Information on age, sex, race, duration of disease, presence of digital ulcers and digit loss, patterns of internal organ involvement, and platelet count was recorded. Cases and controls with a history of renal or hepatic disease, congestive heart failure, active cancer, coronary artery disease, stroke, current pregnancy, and thrombocytopenia were excluded. To avoid the potential confounding effect of an intercurrent infectious process on inflammatory and prothrombotic measures, a standardized questionnaire was used to exclude subjects with signs or symptoms of an upper respiratory tract, urinary tract, dental, or skin infection within the preceding 2 week period[21].

Plasma collection. Antecubital venipuncture without tourniquet was performed after subjects had rested comfortably in a reclining position for 20 min. Plasma samples for measurement of counteradhesive proteins and specific platelet α-granule products were prepared using a modification of the technique of Files, *et al* to minimize *in vitro* platelet activation[22]. The first 2 ml blood was discarded, after which venous samples (4 ml) were collected directly into a precooled plastic syringe containing 1 ml acid-citrate-dextrose solution (NIH Formula A), 10 ml aspirin (180 mg/ml ethanol), and 1 μM prostaglandin E (100 μg/ml ethanol), and inverted gently 5 times, and platelet-poor plasma was prepared by centrifugation (4°C) within 30 min. Blood samples for measurement of soluble E-selectin were collected in citrate anticoagulant (0.11 mol/l, 9:1 vol). All samples were stored at –70°C until assayed in duplicate, with maximum of one freeze-thaw cycle permitted.

ELISA for SPARC, TSP-1, ß-thromboglobulin, PF4, and soluble E-selectin. Plasma samples were assayed for SPARC using a competitive ELISA (Hematological Technology Inc., Essex Junction, VT, USA). TSP-1 was similarly measured by ELISA (American Bioproducts Co., Parsippany, NJ, USA). Plasma samples were immunoassayed for the specific platelet α-granule release products ßTG and PF4 (American Bioproducts). Although plasma for measurement of the counteradhesive and platelet α-granule proteins was collected directly into a solution of platelet inhibitors, the ratio of ßTG to PF4 concentrations was calculated for each subject as a measure of *in vitro* platelet activation[23]. Finally, samples were immunoassayed for soluble E-selectin (R&D Systems, Minneapolis, MN, USA)[5]. In contrast to the other measures, plasma TSP-1 levels were analyzed only in the initial 29 patients with SSc and 11 controls enrolled in the study.

Statistical methods. Data distribution was examined for normality. Log transformation was applied when appropriate. Mean values among cases and controls were compared using Student's t test. Simple and multiple regression analyses were used to examine the relationship between the levels of counteradhesive proteins to ßTG, PF4, and soluble E-selectin. $P < 0.05$ was considered significant using a 2 tailed test.

RESULTS

Demographic and clinical features among the cases with scleroderma and controls are given in Table 1. There were no significant differences by age and sex distribution between cases and controls or between limited and diffuse subsets of scleroderma. There were more non-white participants among cases than controls. In addition, the proportions of participants with digital ulcers as well as disease duration were comparable among the 2 SSc groups. Of note, anticentromere antibody seropositivity was found in a small number of cases, exclusively derived from the limited SSc group. In addition, among the 45 patients with scleroderma, 25 (56.0%) manifested pulmonary, 12 (26.7%) cardiac, 3 (6.7%) renal, 8 (17.8%) muscular, 21 (50.0%) articular, and 40 (88.9%) gastrointestinal disease involvement.

The mean (± SE) SPARC concentration for all SSc patients (103.1 ± 7.4 ng/ml) was increased compared to

Table 1. Demographic and clinical features among scleroderma cases and healthy controls*.

Variable	Healthy Controls, n = 22	Limited SSc, n = 26	Diffuse SSc, n = 19
Mean age ± SE, yrs	4.0 ± 1.8	51.6 ± 2.2	51.4 ± 3.2
Sex, F:M	19:3	24:2	18:1
Race, white:black:other	21:1	19:7	10:8:1
Mean diffuse duration ± SE, yrs	—	10.2 ± 1.7	6.9 ± 1.7
Digital ulcers	—	2	7
Digit loss	—	1	1
Mean number of platelets ± SE ($\times 10^3$)	—	242.9 ± 13.5	291.0 ± 24.1

* No significant differences ($p < 0.05$) exist between any groups or categories.

controls (66.8 ± 8.0 ng/ml; p = 0.0035) (Figure 1). In contrast, the mean SPARC concentration among cases with diffuse SSc (74.1 ± 7.9 ng/ml) was not (p = 0.5). When examined further by disease type, only the mean SPARC level in the limited SSc group (124.0 ± 9.6 ng/ml) was increased compared to controls (p < 0.0001).

In the 29 initial consecutively enrolled cases, the mean plasma TSP-1 level was higher among SSc cases than controls (2.98 ± 0.12 vs 2.40 ± 0.21 ng/ml; p < 0.02; log transformed data, Table 2). Moreover, in the limited SSc patients (n = 20), the mean plasma TSP-1 level (3.02 ± 0.48 ng/ml) was increased compared to controls (p = 0.007). In contrast to the findings for the counteradhesive protein SPARC, mean concentrations of TSP-1 were elevated among both the cases with limited and those with diffuse subsets of SSc. Of note, plasma TSP-1 levels were comparable among SSc patients with limited and diffuse disease (p = 0.7).

Mean (± SE) plasma ßTG concentrations were elevated in the SSc cases compared to controls (150.4 ± 9.2 vs 69.8 ± 14.1 ng/ml; p < 0.0001). Similar elevations were observed in both the limited and diffuse SSc groups (Table 2). Levels of PF4, the other platelet α-granule product, were also higher among cases, both those with limited and diffuse disease, relative to controls (71.2 ± 5.6 vs 30.2 ± 7.7 ng/ml; p < 0.0001). No significant differences were observed in the concentrations of ßTG and PF4 between the limited and diffuse SSc subsets.

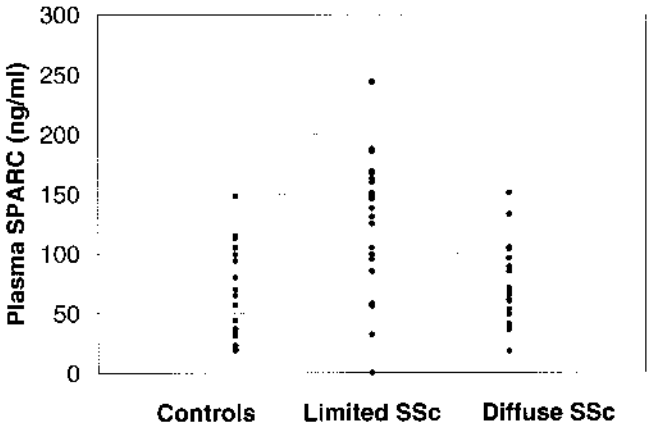

Figure 1. Plasma concentrations of SPARC. Each symbol represents plasma concentration (ng/ml) of SPARC in an individual. Mean concentrations in healthy controls (n = 22), in patients with limited SSc (n = 26), and in patients with diffuse SSc (n = 9) are presented in Table 2.

Regression analysis revealed a strong positive relationship between concentrations of plasma ßTG and PF4 for controls (r = 0.97, p < 0.0001) as well as among SSc cases (r = 0.79, p < 0.0001), indicating internal consistency for these 2 distinct platelet α-granule release products. The mean (± SE) ßTG/PF4 ratios in controls and patients with limited and diffuse SSc were 3.39 ± 1.87, 2.93 ± 1.96, and 2.66 ± 2.31, respectively. The mean ßTG/PF4 ratios between these 3 groups were not significantly different (p > 0.2).

To investigate whether the observed higher concentrations of SPARC in the limited SSc group could be related to *in vivo* platelet activation, the relationships between circulating levels of SPARC to ßTG and to PF4 were evaluated using simple regression analysis. No significant relationships between the levels of plasma SPARC and either ßTG (r = 0.11, p = 0.61) or PF4 (r = 0.18, p = 0.41) levels were found. Plasma SPARC levels were also unrelated to these specific platelet release products in the diffuse SSc and control groups. In contrast to SPARC, regression analysis revealed that plasma TSP-1 levels in the SSc patients had a strong positive relationship with plasma concentrations of ßTG (r = 0.57, p = 0.0014) and PF4 (r = 0.41, p = 0.026).

The mean (± SE) log transformed E-selectin concentrations in all SSc patients (4.05 ± 0.08 ng/ml), limited SSc patients (4.07 ± 0.10 ng/ml), and diffuse SSc patients (4.02 ± 0.13 ng/ml) each were elevated compared to controls (3.54 ± 0.10 ng/ml) (Table 2). However, elevations in E-selectin levels were of similar magnitude in the limited and diffuse SSc groups. As with the platelet α-granule products, we examined the relationship of plasma levels of SPARC to E-selectin levels by simple regression analysis. This revealed that in the limited SSc group, SPARC levels were unrelated to E-selectin levels (r = 0.19, p = 0.38).

DISCUSSION

We demonstrate that plasma concentrations of 2 structurally distinct but functionally overlapping counteradhesive proteins, SPARC and TSP-1, are elevated in patients with scleroderma. TSP-1 levels were increased in patients with limited and diffuse SSc, whereas elevations of SPARC were restricted to the limited SSc subset.

Endothelial cell activation and platelet degranulation are both implicated in the pathogenesis of SSc vasculopathy[4,7,8]. Both endothelial cells and platelets may serve as sources for the 2 counteradhesive proteins we studied[9-11]. Therefore, we measured the concentrations of soluble E-selectin, as a

Table 2. Circulating levels of counteradhesive proteins and markers of platelet and endothelial activation among cases with limited and diffuse scleroderma and healthy controls*.

Measure	Healthy Controls	Limited SSc	Diffuse SSc
SPARC	66.8 ± 8.0	124.0 ± 9.6‡	74.6 ± 7.9
TSP-1†	13.9 ± 3.2	23.2 ± 2.7	27.2 ± 8.5
Log transformed	2.4 ± 0.21	3.02 ± 0.49‡	3.02 ± 0.49‡
2.90 ± 0.33‡			
ßTG	69.8 ± 14.1	160.0 ± 10.6‡	137.2 ± 16.1‡
PF4	30.2 ± 7.7	75.1 ± 6.9‡	65.8 ± 9.4‡
E-selectin	37.8 ± 3.1	69.8 ± 6.5	64.9 ± 8.9
Log transformed	3.54 ± 0.10	4.07 ± 0.10‡	4.02 ± 0.13‡

* Values expressed as mean ± SE (ng/ml), unless otherwise indicated.
† TSP-1 was only assayed in the initial 29 SSc patients and 11 controls enrolled in the study.
‡ Result significantly increased compared to controls at $p < 0.05$.

marker of endothelial cell activation, and of ßTG and PF4, as markers of platelet α-granule release, and compared them to circulating concentrations of the 2 counteradhesive proteins. Mean plasma levels of E-selectin, ßTG, and PF4 were each elevated among patients with SSc, in both the limited and diffuse subsets, in comparison to controls. However, elevated concentrations of SPARC appeared unrelated to measured indices of both platelet α-granule release and endothelial cell activation. In contrast, plasma TSP-1 concentrations were positively related to the markers of platelet α-granule release. We note that 17 of the 45 (37.8%) scleroderma patients were receiving aspirin or nonsteroidal antiinflammatory drug (NSAID) therapy. Since these agents inhibit the cyclooxygenase pathway and platelet activation, use of such therapy could have diminished platelet release of α-granule products including SPARC, TSP-1, ßTG, and PF4 in the SSc group. Thus the increased concentrations of circulating proteins observed in SSc patients, especially those that tightly correlated with established platelet products, may have been even more elevated in the absence of aspirin or NSAID therapy.

SPARC and TSP-1 are each known to be expressed in multiple host tissues relevant to SSc vasculopathy, including vascular endothelial and smooth muscle cells, platelets, and fibroblasts[9-11]. Several mechanisms have been proposed for the observed endothelial cell injury in SSc, including increased serum protease activity that is cytotoxic for endothelial cells[24,25]. Of interest, SPARC induces multiple proteases including collagenase, stromelysin, 92 kDa gelatinase, and plasminogen activator inhibitor-1 (PAI-1)[13,26]. Similarly, TSP-1 reportedly increases 2 leukocyte derived serine proteases, elastase and cathepsin G[27]. Another early event in the pathogenesis of SSc vasculopathy is the perivascular recruitment of leukocytes[3]. Although this has been explained, in part, through an interleukin 1 dependent increase in endothelial cell surface expression of adhesion molecules[28], SPARC and TSP-1 each have been shown to open the endothelial paracellular pathway[12-14] through which leukocyte diapedesis occurs[29], and TSP promotes leukocyte motility *in vitro*[30]. Interestingly, interleukin 1 also reportedly increases SPARC expression[10].

Our findings of increased circulating levels of platelet α-granule products corroborate reports of *in vivo* platelet activation and a prothrombotic state in patients with SSc[7,8]. These findings have included increased intravascular platelet aggregates[3,7], increased circulating ßTG, PF4 and fibrin degradation products[7,8,31], and increased thromboxane synthesis measured as urinary metabolites[32]. SPARC and TSP-1 colocalize within the α-granules of platelets[10,11] and have been shown to preferentially bind to each other[10]. Whether SPARC or TSP-1 causally contributes to the *in vivo* platelet activation seen in SSc is not known. Another limitation of our study is the cross sectional aspect of the data collection. The circulating levels of both SPARC and TSP-1, and levels of ßTG, PF4, and E-selectin, were each measured at a single time point. Thus, we are unable to determine the temporal relationship among these measures. Whether the observed increased circulating concentrations of the counteradhesive proteins represent the initial or a more distal step in the pathway toward endothelial cell dysfunction is not known.

Another component of SSc vasculopathy involves dysregulated fibroblast activity associated with fibrosis and increased deposition of ECM proteins[1-3]. These events have been explained, in part, through increased mitogenic activity for fibroblasts in the plasma of patients with SSc[33] and increased transforming growth factor-ß (TGF-ß) expression in skin[34]. TGF-ß is released by most host tissues in an inactive, latent form[35,36]. TSP-1, which contains specific sequences that can bind to and activate TGF-ß[37-39], is a key regulator of TGF-ß activation *in vivo*[40]. TGF-ß is known to inhibit the endothelial cell proliferative response during wound repair[41]. Interestingly, TGF-ß increases both SPARC and TSP-1 expression[10,11,42]. An example of increased SPARC expression in human fibrotic disease is hepatic

fibrosis[43]. Whether either SPARC or TSP-1 is mechanistically involved in the fibrosis and disordered ECM deposition associated with SSc is unclear.

A late finding in SSc vasculopathy is endothelial cell necrosis, vascular disorganization, and loss of microvessels[1-3]. SPARC and TSP-1 both are increased in response to endothelial cell injury as well as during wound healing[10,11,44]. TSP-1 can directly induce endothelial cell apoptosis[45]. Exogenous SPARC inhibits endothelial cell cycle progression and TSP-1 inhibits angiogenesis[10,11]. While retarding angiogenesis, the 2 counteradhesive proteins promote fibroblast and vascular smooth muscle cell proliferation[10,11]. Both proteins induce endothelial cell actin reorganization and focal adhesion disassembly[9-12]. The abilities of these 2 counteradhesive proteins to perturb endothelial cell-matrix and endothelial cell-cell interactions, endothelial cell cytoskeletal organization and morphology, ECM protein and growth factor function, endothelial cell proliferation, and metalloprotease expression, all support their potential involvement in the disordered vascular repair and remodeling seen in response to the SSc disease state.

On the basis of this case-control study, we propose that increased SPARC and TSP-1 expression may well be operative during one or more host responses that contribute to SSc vasculopathy, including the initial endothelial cell response to an as yet undefined stimulus, perivascular edema and recruitment of leukocytes, *in vivo* platelet activation and aggregation, dysregulated fibroblast proliferation and ECM protein synthesis, and reduction of microvessels. Increased SPARC expression was restricted to the limited form of SSc, whereas elevated TSP-1 was observed in both disease subsets. Finally, increased circulating levels of soluble E-selectin and PF4 may contribute in concert with the 2 counteradhesive proteins to the disordered endothelial cell response to the SSc disease state. That circulating SPARC and TSP-1 are elevated in patients with SSc raises the possibility that counteradhesive proteins, which regulate vascular organization and remodeling in response to endothelial cell injury, might contribute to the pathogenesis of SSc vasculopathy. Additional studies are needed to determine whether these counteradhesive proteins represent surrogates of disease activity or severity, or whether they are causally related to SSc vasculopathy.

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