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Kidney International, Vol. 68 (2005), pp. 110–120

Shedding of endothelial protein C receptor contributes to vasculopathy and renal injury in lupus: In vivo and in vitro evidence[1]

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Shedding of endothelial protein C receptor contributes to vasculopathy and renal injury in lupus: In vivo and in vitro evidence.

Background. Candidate biomarkers for vasculopathy in systemic lupus erythematosus (SLE) include circulating endothelial cells and the recently identified endothelial protein C receptor (EPCR) which, when shed, promotes a thrombotic diathesis. This study sought correlation between plasma levels of soluble EPCR and disease manifestation/severity, with a focus on lupus nephritis.

Methods. In 81 SLE patients (evaluated cross-sectionally and longitudinally) and 59 healthy controls, levels of soluble EPCR and soluble E-selectin were assessed by sandwich enzyme-linked immunosorbent assay (ELISA), circulating endothelial cells isolated by immunomagnetic separation, and EPCR gene polymorphisms determined. Mechanisms of vascular injury were addressed in vitro in human aortic endothelial cells (HAEC) cultured in the presence and absence of interferon-γ (IFN-γ).

Results. The mean level of soluble EPCR was significantly higher in SLE patients (263 ± 13 ng/mL) than controls (174 ± 11 ng/mL) ($P < 0.0001$). Patients with active or past renal involvement had significantly higher mean soluble EPCR levels (306 ± 21 ng/mL) ($N = 40$) than patients without nephritis (228 ± 14 ng/mL) ($N = 41$) ($P = 0.0033$). Mean soluble EPCR correlated positively with serum creatinine ($R = 0.3429$, $P < 0.0001$). The prevalence of the enhanced-shedding EPCR polymorphism A6936G was higher in SLE (41%) ($N = 27$) than controls (7%) ($N = 29$) ($P = 0.0039$). Patient and control plasma were also interrogated for soluble E-selectin, a comparator plasma marker. The results suggest that soluble E-selectin and soluble EPCR are not equivalent end points of vasculopathy and endothelial perturbation in SLE. Although in SLE patients the absence or diminished expression of membrane EPCR on circulating endothelial cells varied, the rare circulating endothelial cells detected in controls invariably expressed membrane-bound EPCR. IFN-γ–treated HAEC expressed less membrane-bound EPCR [133 relative fluorescence units (rfu)] than untreated HAEC (275 rfu); more soluble EPCR was detected in IFN-γ–treated (1.1 ng/10^6 cells) than untreated HAEC (0.65 ng/10^6 cells) ($P = 0.027$).

Conclusion. The results obtained from this cross-sectional/longitudinal study support the hypothesis that the vascular dysfunction characteristic of SLE may be related to a dramatically altered distribution of EPCR, both soluble and membrane-bound forms.

Organ-specific manifestations in systemic lupus erythematosus (SLE) are highly influenced by the inherent characteristics of the vasculature. The endothelium normally functions to thwart cell extravasation, but at inflammatory sites this living barrier undergoes remarkable phenotypic changes such as expression of adhesion molecules, which facilitate the passage of mononuclear cells. Diverse studies in patients with SLE have confirmed the permissive role of vascular adhesion molecules in the pathogenesis of vasculitis and glomerulonephritis [1–6]. Widespread activation of the endothelium has been suggested by the observation that even in nonlesional, nonsun-exposed (buttock) skin from patients with active SLE, endothelial expression of adhesion molecules as well as inducible nitric oxide synthase (iNOS, NOS2) is up-regulated [7, 8]. These findings support the notion that, in SLE, the vascular endothelium in general is "primed" for injury by activated leukocytes and yet there is no overt injury. When another factor is superimposed on widespread priming, vascular lesions develop, contributing to specific organ injury. For example, the deposition of immune complexes in renal tissue initiates a sequence that ultimately

[1]**See Editorial by Leibovich, p. 407.**

Key words: circulating endothelial cells, endothelial cell protein C receptor, metalloprotease, nephritis, systemic lupus erythematosus, vasculopathy.

Received for publication August 30, 2004
and in revised form December 20, 2004
Accepted for publication January 31, 2005

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involves macrophages, which are recruited to the "primed" endothelium where they secrete inflammatory cytokines such as interferon-γ (IFN-γ) [9].

A systematic study, which examines endothelial injury in the context of known risk factors for disease, is difficult due to the inaccessibility of the endothelium in humans. However, circulating endothelial cells may serve as an important biomarker since they reflect biologic events occurring at sites where the endothelium has been activated and engaged in inflammation with subsequent loss of functional integrity [10].

The endothelial cell protein C receptor (EPCR), a recently identified cell-surface protein with important roles in coagulation and inflammation, inhibits thrombosis by augmenting thrombin-thrombomodulin-dependent protein C activation [11]. Additionally, the shedding of EPCR is associated with the activation of protease-activated receptors (PARs), a novel family of G protein–coupled receptors that are constitutively expressed on endothelial cells and are involved in the early recruitment of leukocytes [12]. While the different factors that regulate the activity and expression of EPCR are still under investigation, recent studies have demonstrated important molecular and genetic influences. In particular, cleavage of the EPCR molecule near the transmembrane domain decreases cell surface expression of EPCR and increases plasma levels of its soluble form, soluble EPCR [13]. Susceptibility to proteolytic cleavage has been shown to be conferred by a genetic polymorphism in exon 4 of the EPCR gene [14]. Such shedding of membrane EPCR would be expected to have a negative impact on endothelial integrity, and on the delicate balance of coagulation and inflammation.

Accordingly, this study was initiated to examine whether plasma levels of soluble EPCR, representative of a dysregulated protein C pathway, are associated with disease manifestation and severity in SLE patients. Confirmation of such an association would be a first step toward identification and subsequent validation of a novel biomarker of vasculopathy. A further prediction is that this candidate biomarker will stratify with disease manifestations more likely to reflect perturbation of vascular integrity. To accomplish this goal, a cross-sectional and longitudinal observational study of SLE patients was conducted in which soluble EPCR levels were measured over time and compared to levels in a healthy control group. Both groups were also genotyped for the appropriate polymorphism on exon 4 of the EPCR gene. In addition, EPCR expression by circulating endothelial cells isolated from SLE patients and healthy controls, as well as by human aortic endothelial cells (HAEC), was evaluated in vitro after stimulation with IFN-γ and interleukin (IL)-1 (cytokines important in the pathobiology of renal disease in SLE).

METHODS

Subjects

This study and its informed consent form were approved by the Institutional Board of Research Associates of NYU School of Medicine. Eighty-one patients who fulfilled at least four of the American College of Rheumatology criteria for the diagnosis of SLE [15] (seen in consultation by the Rheumatology Service as inpatients or outpatients at the Lupus Clinic in the Hospital for Joint Diseases or private practice) were recruited for this study. Patients were enrolled as a convenience sample (in order of presentation to the physician) with the intent to represent a spectrum of disease activity and manifestations. The patient was asked to indicate his or her intention to receive ongoing rheumatologic care, which permitted observational follow-up in the majority of patients (50 patients had $\geq$ two visits; 31 had only one visit). The frequency of patient encounters and the medical management were dictated solely by clinical need.

At the majority of visits, clinical disease activity was assessed by the SELENA Systemic Lupus Erythematosus Disease Activity Index (SLEDAI), an adaptation of the initial SLEDAI which scores ongoing as well as recurrent or new activity [16, 17]. Medications taken and ongoing organ involvement were recorded, and ethylenediaminetetraacetic acid (EDTA)–treated samples of venous blood were collected for laboratory-based studies (see below). Patients' available medical records were reviewed retrospectively to capture prior organ involvement that may have been quiescent during the longitudinal observation period. For the 50 patients with $\geq$ two visits, there was a mean of 4.2 temporally distinct visits (SD 2.5 visits, range 2 to 13 visits), taking place over a mean of 15.8 months (SD 8.8 months, range 1 to 29 months). Fifty-nine healthy subjects were recruited from the personnel of the Hospital for Joint Diseases/NYU School of Medicine. The characteristics of patients and controls at the time of enrollment are summarized in Table 1. Day 0 was defined as the day of first clinical and laboratory evaluation of a given enrolled patient or control.

Determination of plasma soluble EPCR and of plasma soluble E-selectin

Plasma was collected in EDTA during the course of a hospitalization or routine clinic visit. To avoid venipuncture-associated circulating endothelial cells, the blood sample used for this specific analysis was not the first drawn. Soluble EPCR levels were determined by a commercially available enzyme-linked immunosorbent assay (ELISA) (Diagnostica Stago Inc., Parsippany, NJ, USA). The lower limit of detection of this assay is 1 ng/mL. Soluble E-selectin levels were determined by

Table 1. Baseline characteristics of study subjects

	Patients ($N = 81$)	Controls ($N = 59$)
Gender *number* (%)		
Female	69 (85)	50 (85)
Male	12 (15)	9 (15)
Ethnicity *number* (%):		
African American	18 (23)	6 (10)
Asian	7 (8)	17 (29)
Hispanic	46 (57)	10 (17)
White	10 (13)	26 (44)
Age *years*		
Median	39	33
Range	19–61	1–61
SELENA-SLEDAI		
Median score	2	
Range	0–22	
Medications taken by patients *number*		
Azathioprine	23	
Cyclophosphamide	2	
Hydroxychloroquine	56	
Methotrexate	2	
Mycophenalate mofetil	12	
Prednisone	42	
Median dosage	10 mg/day	
Dosage range	5–60 mg/day	
Warfarin	14	

a commercially available ELISA (R&D Systems, Minneapolis, MN, USA). To eliminate day-to-day interassay variability, all plasma samples from a given patient were stored at −20°C and assayed on the same day without knowledge of SELENA-SLEDAI score or prior organ involvement.

The normal range of plasma soluble EPCR was defined as the geometric mean (+2 SD) of all values obtained over time from the 59 control subjects.

Evaluation of EPCR polymorphism

DNA was isolated from anticoagulated blood using the Qiagen kit (Valencia, CA, USA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) primers were matched to accession number AF375468 of the EPCR gene as previously described [14]. This method uses a PCR restriction-fragment length polymorphism (PCR-RFLP) to genotype the single nucleotide polymorphism (SNP) at exon 4 in each patient. In brief, PCR reactions consisted of 50 to 100 ng DNA in 1 μL Tris EDTA, 2 μL 10 mmol/L deoxynucleoside triphosphate (dNTP), 10 μL 10× Taq buffer, 3 μL 50 mmol/L $MgCl_2$, 5 μL formamide, 10 μL 3 μmol/L 5′-CCTACACTTCGCTGGTCCTGGGCGTCCTGGTCT GC-3′ (EPCR F-6083), 10 μL 3 μmol/L 5′-CAAGTA CTTTGTCCACCTCTCC-3′(EPCR R-6372), 1 μL AmpliTaq polymerase in a final volume of 100 μL. Cycling conditions were as follows: 94°C for 5 minutes; 40 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute; and 72°C for 10 minutes. When complete, 8 μL of product was digested with 1 μL restriction enzyme buffer and 1 μL of PstI overnight at 37°C. Products were run on a 10% acrylamide TBE gel. The high-shedding homozygote (G/G) has only uncut DNA. A heterozygote has one uncut and one cut fragment at 290 and 254 bp, respectively. An individual homozygous for A has only cut fragments. In the case of a patient who was assigned A/G heterozygote, an uncut PCR amplified product was inserted into a plasmid via TA cloning, which was followed by confirmation that the population of clones contained G and A alleles using a commercial DNA sequencing service (GeneWiz, Rutherford, NJ, USA).

Culturing of HAEC

HAEC (Clonetics Corp., San Diego, CA, USA) were cultured in endothelial cell growth media (EGM-2; Clonetics Corp.) in 24-well tissue culture plates. Cultures were seeded at 20% confluency on tissue culture dishes coated with 0.1% gelatin, media changed every 2 to 3 days, passaged via trypsin/EDTA and harvested once per week. Endothelial cells between passages 4 and 8 were used in the experimental conditions described immediately below. Human IL-1 and IFN-γ were obtained from R&D Systems, Inc. and Sigma Chemical Co. (St. Louis, MO, USA), respectively. In brief, HAEC were plated at 50% to 60% confluence on 6-well culture plates (Falcon) in EGM-2 media + 10% fetal bovine serum (FBS). After 24 hours, cells were treated with either IL-1 (5 ng/mL) or IFN-γ (1 μg/mL) in the presence or absence of the metalloprotease inhibitor CC1000 (EMD Biosciences, Inc., San Diego, CA, USA) (10 nmol/L) for 24 hours. Supernatants were collected and assayed for soluble EPCR. Cells were collected, stained with primary antibodies reactive to human EPCR (HEPCR 1489) [18] and antimouse IgG fluorescein isothiocyanate (FITC), and analyzed by FACS (Facs-Scan) (Becton Dickinson, Franklin Lakes, NJ, USA).

Isolation and phenotypic evaluation of circulating endothelial cells

Isolation of circulating endothelial cells was performed by immunomagnetic separation after an antibody incubation step. Whole blood was diluted using phosphate-buffered saline (PBS)/EDTA/bovine serum albumin (BSA) (1:2.5) (PBS + 1 mmol/L EDTA, and 0.5% BSA), incubated for 30 minutes at 4°C with antiendothelial cell monoclonal antibody P1H12 at a 1:200 dilution (16985) (Chemicon, Temecula, CA, USA) conjugated to magnetic beads, and separated using a Dynal MPC-l magnetic particle concentrator (Dynal, Oslo, Norway). The endothelial cell isolate was then suspended in PBS control and affixed to glass coverslips (three coverslips for immunostaining) and to silica (for use in scanning electron microscopy) using a cytospin technique. Regarding the latter, these samples were fixed with 4% paraformaldehyde (20 minutes, 22°C), followed by three washes in PBS, each for 5 minutes at 22°C, and then

stored at 4°C. For immunostaining, circulating endothelial cells applied to glass coverslips were treated with Triton X-100 and stained with anti-EPCR (HEPCR 1489) [18]. Each glass coverslip was incubated for 30 minutes at 22°C at 1:100 with the appropriate antibody or rabbit IgG, followed by three washes in PBS/EDTA/BSA, each for 5 minutes at 22°C. After removal of unbound antibody, a secondary antibody (alkaline phosphatase–conjugated antimouse IgG), was allowed to incubate for 30 minutes at 22°C at 1:100. This was followed by another three washes in PBS/EDTA/BSA, each for 5 minutes at 22°C. Detection of circulating endothelial cells and percent positive EPCR was achieved by sequential treatments with Gill's nuclear stain, and enzyme substrate, respectively. EPCR-positive cells report red (alkaline phosphatase substrate). Circulating endothelial cell phenotype was also assessed based on analysis of cell morphology by scanning electron microscopy. A Leo 982 field emission digital scanning electron microscope was used to measure circulating endothelial cells size and assess surface appearance.

Statistical analysis

The Student *t* test for unpaired data was used to compare soluble EPCR measurements between the different groups. Statistical significance of the differences in frequencies of a specific genotype (G/G, A/G, or A/A) in patients and controls was estimated by Fisher's exact test with the aid of Instat software (GraphPad, San Diego, CA, USA). *P* value <0.05 was considered to be significant. In addition, Spearman's rank correlations between circulating endothelial cell levels and SELENA-SLEDAI were calculated.

RESULTS

Plasma levels of soluble EPCR

As demonstrated in Figure 1, the mean level of soluble EPCR was significantly elevated in the cohort of SLE patients compared to the healthy controls (267 ± 13 ng/mL vs. 174 ± 11 ng/mL, respectively) ($P < 0.0001$). Using soluble EPCR as a phenotype, patients were subgrouped based on levels of soluble EPCR relative to the normal range of plasma soluble EPCR (i.e., controls). For example, in 23 (28%) of the 81 SLE patients (defined as group A), soluble EPCR levels were significantly elevated (>2 SD above control mean) (i.e., >339 ng/mL). By contrast, only three (5%) of 59 control subjects had elevated soluble EPCR.

Overall, there was considerable heterogeneity in the levels of plasma soluble EPCR among the SLE patients. Twenty-three (28%) patients had a significantly elevated level (>339 ng/mL) on at least one visit (group A). Moreover, when soluble EPCR was plotted at each clinic visit in this group, values were highly variable (data given where longitudinal measurements were available) (Fig. 2A). For example, soluble EPCR values of one patient at four visits over a 24-month period were 267, 695, 396, 553 ng/mL (mean 475, SD 188 ng/mL). Twenty-nine (36%) SLE patients exhibited moderately elevated levels of soluble EPCR (201 to 339 ng/mL) (group B). The remaining 29 (36%) patients exhibited persistently normal soluble EPCR levels (group C) equivalent to that seen in the controls (≤200 ng/mL). In groups B, C, and controls, the levels of soluble EPCR remained stable (Fig. 2B to D).

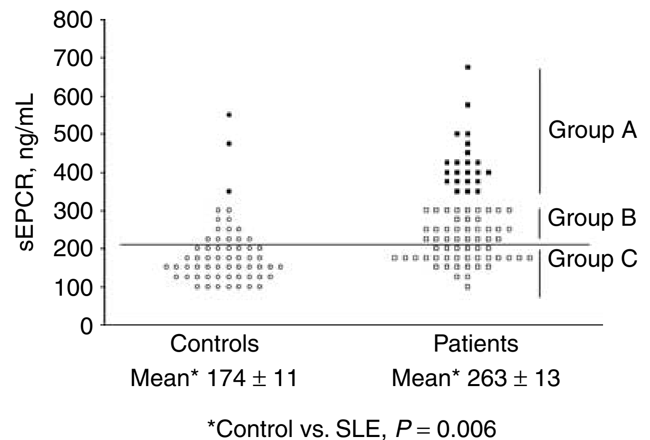

Fig. 1. Plasma levels of soluble endothelial protein C receptor (sEPCR). Distribution of soluble EPCR in 59 healthy controls and 81 systemic lupus erythematosus (SLE) patients. Individuals with soluble EPCR greater than mean control + 2 SD are indicated by bold symbols. Symbols above line indicate soluble EPCR levels greater than those of 75% of the control subjects (i.e., >200 ng/mL).

Analysis of SNP of EPCR gene at nt 6936 (A → G, associated with increased EPCR shedding)

The EPCR polymorphism A6936G (associated with increased EPCR shedding) was analyzed in 27 SLE patients and 29 controls by a method employing PCR and restriction enzyme digestion (Table 2). Eleven (41%) of the 27 SLE patients were heterozygous for the G allele (associated with increased EPCR shedding). In contrast, only two (7%) of the controls were heterozygous for the G allele ($P = 0.0039$, SLE vs. controls). As predicted, the mean levels of soluble EPCR were elevated in both groups with A/G genotype (357 ± 34 ng/mL for patients and 342 ng/mL for controls) (only two of 29 controls were A/G). Of the eight group A patients analyzed, seven (88%) had the A/G genotype, while only one (12%) had the A/A genotype.

Sixteen (62%) of the 27 SLE patients were homozygous for the A allele. In this subgroup, the soluble EPCR level was 225 ± 18 ng/mL, which was significantly higher than in controls with the A/A genotype (147 ± 16 ng/mL) ($P = 0.0032$, SLE vs. control). In aggregate, while the A/G genotype was associated with high levels of soluble EPCR, there were also patients with high levels of soluble EPCR that were not accounted for by their EPCR genotype.

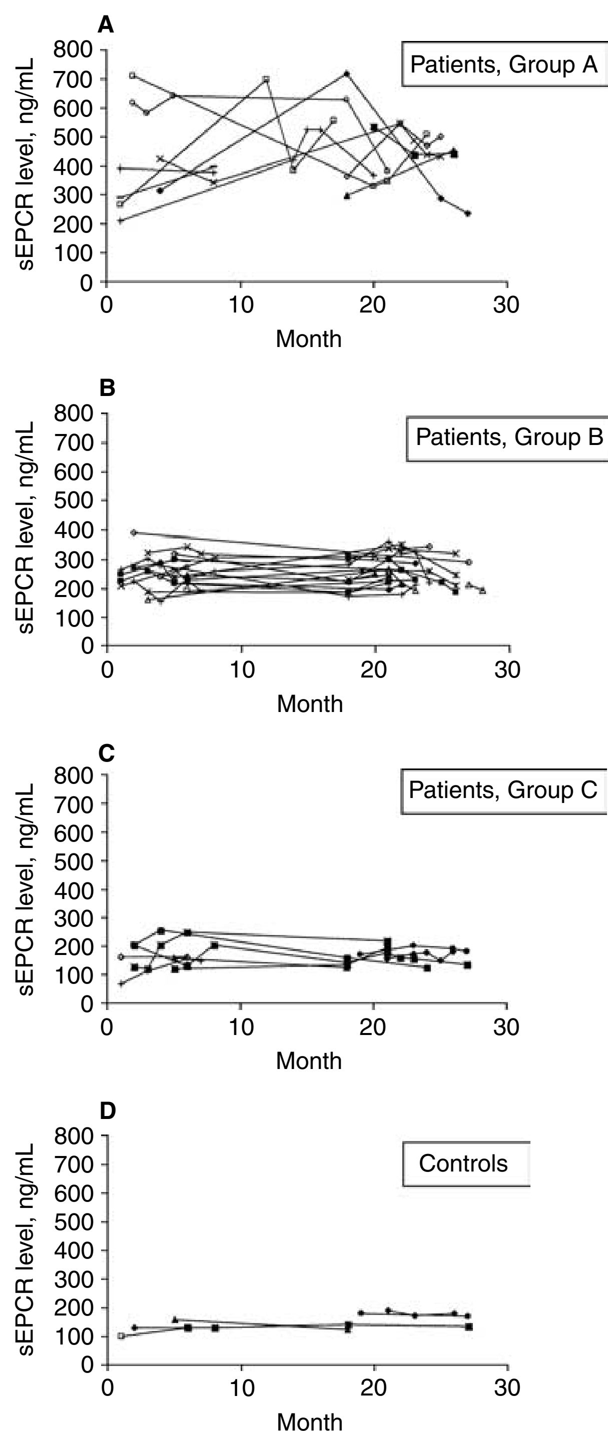

Fig. 2. Serial studies of soluble endothelial protein C receptor (sEPCR) levels in plasma. Plasma levels of soluble EPCR were assessed in systemic lupus erythematosus (SLE) patients (*A* to *C*) and controls (*D*) at separate visits during an interval of 27 months. (A) Subset of SLE patients with soluble EPCR levels > control mean + 2 SD (>339 ng/mL) (group A); data are longitudinal measurements (where available). (B) SLE patients with soluble EPCR >75% of controls but <mean control + 2 SD (group B). (C) SLE patients with soluble EPCR ≤75% of controls (≤200 ng/mL) (group C). (D) Controls. Note: When soluble EPCR was plotted at each clinic visit for patients in group A, values were highly variable.

Association of plasma soluble EPCR but not EPCR genotype with organ involvement

Clinical disease activity (as measured by SELENA-SLEDAI) for any given patient at any point in time was not reliably paralleled by plasma soluble EPCR (not shown). This is not surprising, since SLEDAI does not necessarily reflect endothelial cell injury at a given point in time. To provide clinical data beyond a numerical score on SELENA-SLEDAI, the specific disease manifestations present at each SLEDAI assessment are shown in Table 3. The combined categories of stroke and venous thrombosis were more often seen in patients in groups A and B compared to group C, but the small sample size precluded statistical analysis. Active renal disease at the time of a patient encounter was most often present in group A (group A vs. group C) ($P = 0.011$). The incidence of nephritis, past or present (Table 4), was significantly higher in group A (65%) but not in group B (55%) vs. group C (31.0%) ($P = 0.0244$ and $P =$ NS, respectively).

Patients with nephritis had significantly higher mean levels of plasma soluble EPCR (306 ± 21 ng/mL) compared to patients with no history of nephritis (228 ± 14 ng/mL) ($P = 0.0033$) (Table 4). Patients with nephritis in group A (but not groups B or C) had significantly higher mean levels of plasma soluble EPCR. For example, the soluble EPCR of patients with nephritis (452 ± 24 ng/mL) was significantly elevated when compared to patients with no history of nephritis (381 ± 11 ng/mL) ($P = 0.04$) (Table 4).

In the patients with nephritis, the elevation of soluble EPCR occurred even in the presence of extensive proteinuria (>2 g/day). The nephritis group included a subset of patients who excreted substantial amounts (>2 g) of protein per day at some point during the observation periods. There was also a positive correlation observed ($R = 0.3429$, $P < 0.0001$) when levels of soluble EPCR were compared to levels of serum creatinine. These patient assessments (proteinuria and creatinine) reflect impaired renal function that occurred during the observation period. However, impaired renal function would not account for high levels of soluble EPCR in group A. In one scenario, the assessment of plasma soluble EPCR is an underestimation of total soluble EPCR, where renal excretion of soluble EPCR might have been expected to have lower plasma soluble EPCR. Although the numbers are limited, the presence of the A/G genotype did not invariably associate with nephritis. Five of the 13 patients with nephritis had the A/G genotype, whereas six of the 14 patients without nephritis also had the A/G genotype.

Plasma levels of soluble E-selectin

As shown in Figure 3, the mean level of soluble E-selectin was significantly elevated in the cohort of SLE patients compared to the healthy controls (58.5 ± 4.5

Table 2. Analysis of single nucleotide polymorphism (SNP) of endothelial protein C receptor (EPCR) gene at nt 6936

	Genotype		Mean plasma soluble EPCR by genotype	
	Patients ($N = 27$)	Controls ($N = 29$)	Patients ($N = 27$)	Controls ($N = 29$)
A/A	59%	93%	225 ± 18 ng/mL[a]	157 ± 13 ng/mL
A/G	41%[b]	7%	357 ± 34 ng/mL	342 ng/mL[c]
G/G	0%	0%	—	—

[a]Patients vs. controls, $P = 0.0006$.
[b]Patients vs. controls, $P = 0.0145$.
[c]Only 2 of 29 controls were A/G.

Table 3. Clinical manifestations in systemic lupus erythematosus (SLE) patients at each SELENA-SLEDAI assessment

	Group A ($N = 29$)	Group B ($N = 46$)	Group C ($N = 26$)
Active renal disease	12[a]	7	1
Arthritis	0	6	1
Cranial nerve disorder	1	0	0
Hematologic abnormality	3	3	2
Myositis	3	0	0
Mucocutaneous lesions	3	3	4
Pleurisy/pericarditis	0	0	0
Stroke	2	4	0
Vasculitis	0	0	0
Venous thrombosis	1	1	0

N is the number of SELENA-SLEDAI assessments.
[a]Active renal disease in group A vs. group C, $P = 0.011$.

Table 4. Plasma levels of soluble endothelial protein C receptor (EPCR) in patients with and without history of nephritis

	Number of patients		Plasma soluble EPCR	
	Nephritis	No nephritis	Nephritis	No nephritis
Total	40	41	306 ± 21 ng/mL[a]	228 ± 14 ng/mL
Group A	15	8	452 ± 24 ng/mL[b]	381 ± 11 ng/mL
Group B	16	13	249 ± 8 ng/mL	248 ± 9 ng/mL
Group C	9	20	166 ± 9 ng/mL	153 ± 5 ng/mL

[a]Total patients, nephritis vs. no nephritis ($P = 0.0033$); nephritis 95% CI 264 to 349; no nephritis 95% CI 199 to 257.
[b]Group A patients, nephritis vs. no nephritis ($P = 0.04$); Group A nephritis 95% CI 402–502; Group A no nephritis 95% CI 355–406.

ng/mL vs. 36.5 ± 2.5 ng/mL) ($P < 0.0001$). Since soluble E-selectin represents a specific marker of inflammatory activation of the vascular endothelium [19], patient subgroups were analyzed for soluble E-selectin. There was no difference in soluble E-selectin levels in patients with nephritis versus patients without nephritis (56.5 ± 4 ng/mL vs. 63.3 ± 9 ng/mL) ($P =$ NS). In addition, when soluble E-selectin at each clinic visit was plotted versus soluble EPCR, there was no correlation ($R = 0.006$, $P =$ NS) (Fig. 3B). Moreover, there were no differences in soluble E-selectin in patients of EPCR group A (Fig. 1) or of high EPCR shedding A/G genotype (Table 1) (55.5 ± 5.6 ng/mL and 57.1 ± 7 ng/mL, respectively). Taken together, soluble E-selectin and soluble EPCR are not equivalent end points of vasculopathy and endothelial perturbation in SLE.

Measurements of soluble EPCR after in vitro stimulation of HAEC

The next set of experiments were designed to address the molecular mechanism(s) contributing to the increased levels of soluble EPCR in SLE patients, particularly those with renal involvement, since this observation was not fully accounted for by EPCR genotyping. The approach exploited an in vitro model in which HAEC were treated with inflammatory cytokines likely to be important in the pathobiology of SLE. Specifically, HAEC were cultured in the presence or absence of IFN-γ or IL-1, and in the presence and absence of a serine protease inhibitor (aprotinin) or a selective metalloprotease inhibitor (CC1000). Treatment was followed by retrieval of cells for assessment of expression of membrane-bound EPCR and supernatants for evaluation of soluble EPCR.

As seen in Figure 4A, HAEC incubated with IFN-γ (100 ng/mL, 24 hours) expressed significantly less membrane-bound EPCR than cells incubated with medium alone (133 rfu vs. 275 rfu ($N = 2$) (FACS). Moreover, significantly higher levels of soluble EPCR were detected in the supernatants generated from IFN-γ–treated HAEC than in supernatants from HAEC incubated with medium alone (1102 pg/10^6 cells vs. 657 pg/10^6 cells) ($P = 0.027$) (Fig. 4B). Soluble EPCR was also significantly higher in the supernatants generated following incubation of HAEC with IL-1 (850 pg/10^6 cells) ($P = 0.019$, IL-1 vs. control) (Fig. 4B). The addition of metalloprotease inhibitors (CC1000) substantially reduced the level of soluble EPCR in the supernatants generated under all treatments (control, IFNγ-, and IL-1). Inhibition was 60%, 67%, and 61%, respectively. In contrast, serine protease inhibitors (aprotinin) had no effect on shedding of EPCR.

Evaluation of circulating endothelial cell phenotype in SLE patients and controls

Peripheral blood samples from 28 SLE patients and four controls were used for the simultaneous evaluations of soluble EPCR and circulating endothelial cells (see **Methods** section), which were stained for membrane-bound EPCR. The circulating endothelial cells from the patients were heterogeneous in their expression of membrane-bound EPCR. Cells that did not stain for

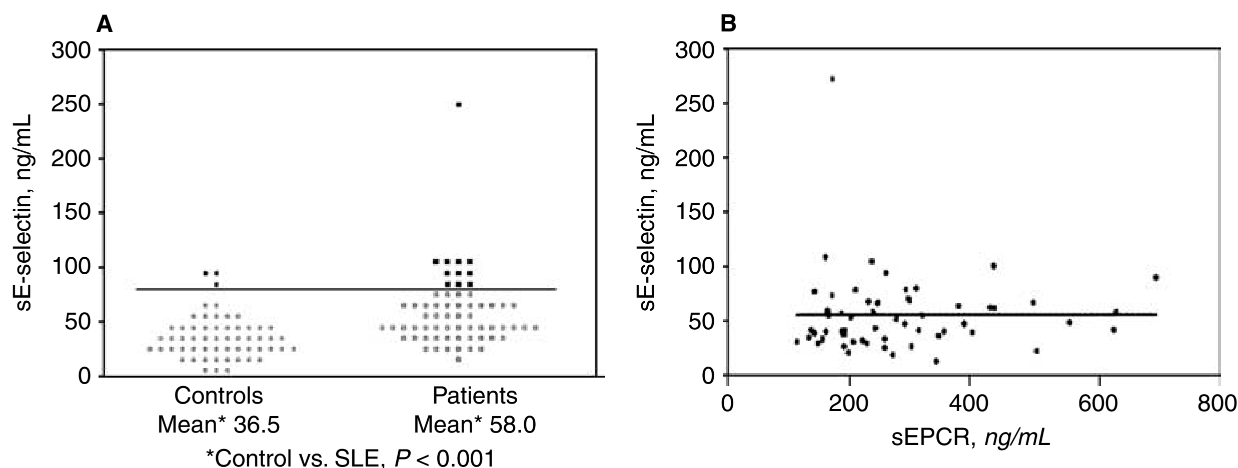

Fig. 3. Plasma levels of soluble E-selectin (sE-selectin). (*A*) Distribution of soluble E-selectin in 57 healthy controls and 63 systemic lupus erythematosus (SLE) patients. Individuals with soluble E-selectin levels greater than mean control + 2 SD are indicated by bold symbols. (*B*) Correlation of soluble E-selectin levels with levels of soluble endothelial protein C receptor (sEPCR).

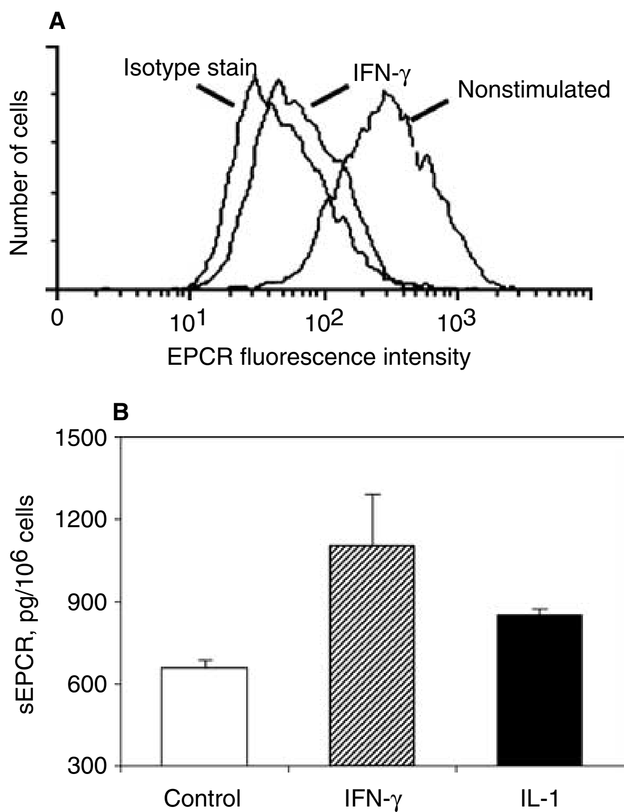

Fig. 4. Membrane-bound endothelial protein C receptor (mEPCR) in human aortic endothelial cells (HAEC). HAEC were incubated with or without interferon-γ (IFN-γ) (100 ng/mL, 24 hours), interleukin (IL)-1 (5 ng/mL) in the absence and presence of metalloprotease inhibitors (CC1000, 10 nM). Resting and IFN-γ–treated HAEC were stained with mouse anti-EPCR or isotype and fluorescein isothiocyanate (FITC) antimouse IgG. (*A*) Cells were analyzed by FACS. (*B*) Cell fluids, from the HAEC experiment in (A), were analyzed for soluble EPCR (sEPCR) by a sandwich enzyme-linked immunosorbent assay (ELISA) method.

membrane-bound EPCR (Fig. 5D) as well as cells that stained positive for membrane-bound EPCR (Fig. 5E) were evident in each of the patient isolates; the percentage of circulating endothelial cells that did not express membrane-bound EPCR ranged from 6% to 70%. The percentage of total circulating endothelial cells containing membrane-bound EPCR did not correlate to disease activity (not shown, $R = 0.2419$, $P =$ NS). In addition, the percentage of membrane-bound EPCR-positive circulating endothelial cells in patients did not correlate with soluble EPCR levels (not shown, $R = 0.1524$, $P =$ NS). Therefore, in contrast to the in vitro studies of HAEC, in which we observed a loss of membrane-bound EPCR and concomitant retrieval of soluble EPCR in cell supernatants, such a clearly reciprocal relationship was not observed in vivo.

Circulating endothelial cells isolated from controls consistently stained for membrane-bound EPCR (i.e., 0% EPCR-negative cells) (see Fig. 5F). Circulating endothelial cells stained appropriately with isotype control (Fig. 5C). Consistent with our previous observation [10], the levels of circulating endothelial cells were significantly higher in SLE patients than in controls (not shown, $P = 0.02$) and the levels of circulating endothelial cells in patients correlated in directly with SELENA-SLEDAI scores (not shown, $R = 0.80$, $P = 0.001$). Evaluation by scanning electron microscopy indicated that circulating endothelial cells isolated from both controls and SLE patients exhibited the morphology characteristic of cells derived from the endothelium (Fig. 5A and B, respectively).

The medications taken by the SLE patients (as subdivided into groups A to C) are listed in Table 5. Prednisone, hydroxychloroquine, and azathioprine were the most widely used, while patients were less likely given warfarin, mycophenolate mofetil, cyclophosphamide, and

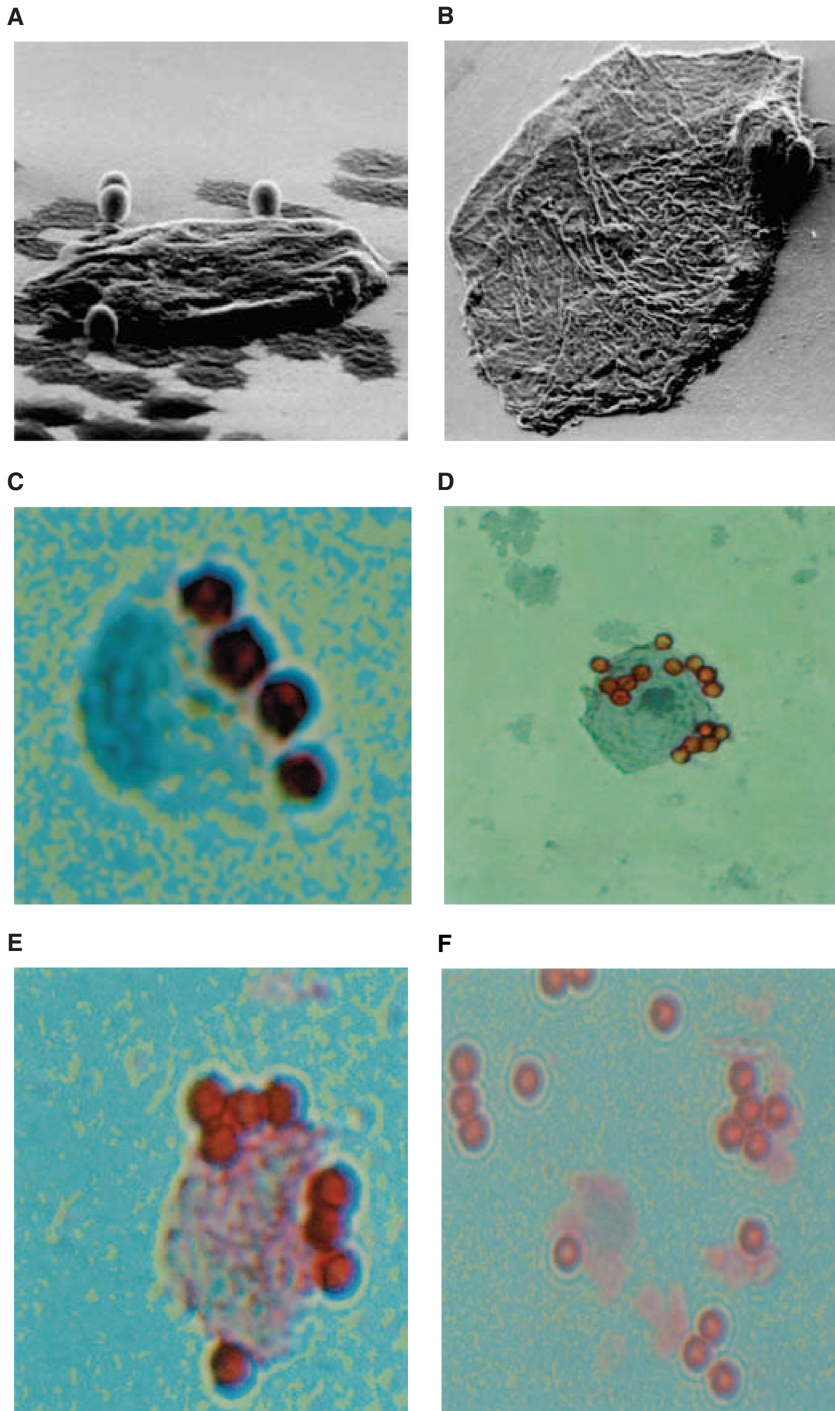

Fig. 5. Membrane-bound endothelial protein C receptor (mEPCR) in circulating endothelial cells from systemic lupus erythematosus (SLE) patients and controls. Circulating endothelial cells were isolated from peripheral blood as described. Representative stains of a patient with SLE (*B* to *E*) and controls (*A* and *F*). Venous blood was drawn into tubes with ethylenediaminetetraacetic acid (EDTA) and studied immediately. Circulating endothelial cells were isolated by immunomagnetic separation using P1H12-coated iron beads. The cell isolate was applied to silica and analyzed by scanning electron microscopy (A and B) or applied as a cytospin to a slide, fixed, stained with isotype control (C) or with anti-EPCR (D to F) and alkaline phosphatase–conjugated antimouse IgG, counterstained with hematoxylin. (D) A representative EPCR-negative circulating endothelial cell from an SLE patient. (E and F) Cells that stain positive for EPCR from SLE patient (E) and control (F).

methotrexate. There were no significant treatment differences among the groups. However, the daily prednisone dosage was significantly higher in groups A and B vs. group C ($P = 0.05$, $P = 0.0075$, respectively).

DISCUSSION

The vasculature not only provides a dynamic boundary between potential inflammatory mediators in the circulation and the cellular components of the tissues, but also contributes to the dynamic regulation of coagulation. In SLE, barrier function may be compromised since a hallmark of this disease is the recurrence of widespread and diverse vascular lesions. The EPCR, poised to limit exaggerated inflammation as well as coagulation, represents a potentially novel participant in the pathobiology of human SLE. The results obtained from this cross-sectional/longitudinal study support the hypothesis that

Table 5. Medications of systemic lupus erythematosus (SLE) patients at each visit

	Group A ($N = 23$)	Group B ($N = 29$)	Group C ($N = 29$)
Azathioprine	9	15	6
Cyclophosphamide	2	0	0
Hydroxychloroquine	19	31	22
Methotrexate	0	3	0
Mycophenalate mofetil	7	8	1
Prednisone	21	16	15
Mean daily dose[a]	25 ± 5 mg	29 ± 6 mg	8 ± 1 mg
Warfarin	5	12	1

N is the number of patients in each group. Number of medications reflects each medication noted for each patient at each visit. Note: the number and frequency of patient visits and medical management were dictated solely by clinical need.

[a]Mean daily dose of prednisone: group A vs. group C ($P = 0.05$); group B vs. group C ($P = 0.0075$).

the vascular dysfunction characteristic of SLE may be related to a dramatically altered distribution of EPCR, both soluble and membrane-bound forms, as evidenced by (*1*) increased levels of soluble EPCR in patients with SLE, particularly those with renal disease; (*2*) an enrichment of the high EPCR shedding A/G genotype in SLE; (*3*) decreased expression of membrane-bound EPCR and reciprocal increased soluble EPCR in cytokine-stimulated endothelial cells in vitro; and (*4*) variable expression of membrane-bound EPCR on circulating endothelial cells from SLE patients, but decreased compared to controls.

Circulating endothelial cells have been used as an acceptable surrogate for the study of vessel wall endothelium since they would be subjected to the same blood-borne endothelial-activating influences as vessel wall endothelium. Elevated levels of circulating endothelial cells may be a proxy for vascular injury, as recently demonstrated in patients with sickle cell anemia and in patients with small vessel vasculitis [20, 21]. An increase in tissue factor expression by these cells reflected a potential contribution to coagulation. Interestingly, we showed decreased EPCR expression, indicating that SLE circulating endothelial cells lose an anticoagulant phenotype during the process of detachment.

EPCR, a recently described member of the protein C anticoagulant pathway [11], accelerates formation of activated protein C [22]. Immunohistochemistry studies have shown that membrane-bound EPCR is expressed primarily on endothelial cells [18]. In an atherosclerosis biopsy study involving immunostaining of human coronary artery, EPCR expression was decreased in diseased areas of blood vessel relative to normal-appearing areas [23]. Thrombin and IL-1 have been shown to promote the shedding of EPCR and accumulation of soluble EPCR [13]. Kurosawa et al [24] observed an increase in soluble EPCR in blood obtained from SLE patients and patients with sepsis relative to normal volunteers, suggesting that plasma soluble EPCR may have utility as a biomarker of blood vessel disease. A recent study demonstrated that plasma soluble EPCR inhibits protein C activation in vitro [25], implying that elevated levels of soluble EPCR will promote a thrombotic diathesis.

That endothelial cells are predisposed or "primed" for recruitment of activated leukocytes (e.g., macrophages) in SLE may provide insight into the cellular mechanism(s) contributing to EPCR shedding. In this scenario, macrophages recruited to the endothelium via increased expression of adhesion molecules secrete inflammatory cytokines such as IFN-γ at sites of tissue injury. A consequence might be release of IFN-γ into the circulation. Of relevance, Behrens et al [26] demonstrated increased mRNA expression of genes known to be induced by IFN-γ in peripheral mononuclear cells of SLE patients, most notably in those with renal disease. Further support for a role of IFN-γ in renal disease was provided by Masutani et al [9] who demonstrated high expression of this cytokine, as well as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), in kidney biopsy tissue from patients with diffuse proliferative glomerulonephritis.

Studies in lupus mice also support the notion that the endothelium in renal tissue is exquisitely sensitive to IFN-γ. In the MRL-lpr lupus model, treatment with anti-IFN-γ–specific monoclonal antibody or soluble IFN-γ receptor resulted in a reduction in adhesion molecules and a significant delay in disease progression. Carvalho-Pinto et al [27] provide evidence that MRL-lpr mice genetically deficient in IFN-γ production are capable of generating anti-dsDNA antibodies but do not develop renal disease. Importantly, infusion of these mice with macrophages capable of secreting IFN-γ resulted in the renal injury seen in the wild-type mice [27].

Xu et al [28] showed that thrombin effects on EPCR shedding in the presence and absence of IL-1 were completely eliminated by the hydroxamic-based inhibitor KD-IX-73-4. Similarly, in the present study, the metalloprotease inhibitor CC1000 attenuated IFN-γ– and IL-1–dependent EPCR shedding.

Further investigation is needed to establish whether soluble EPCR is a biomarker of IFN-γ–stimulated endothelial injury in renal tissue, and whether components of the pathway leading to shedding of EPCR (from IFN-γ stimulation to metalloprotease cleavage) may be fruitful targets of therapeutic intervention.

Another mechanism for increased shedding, which may operate in isolation or in concert with the IFN-γ–dependent pathway of soluble EPCR generation, is the genetic predisposition conferred by the G allele at exon 4 of the EPCR gene. Recent evidence has been presented that the A/G genotype leads to elevated soluble EPCR in humans [14]. The mechanism by which the G allele results in shedding of EPCR is unclear, but this SNP codes for a modification of serine 219 to glycine. Recently, Esmon [29] speculated that these residues are in the

transmembrane domain, and in the altered form lead to a glycine-glycine pair that would likely alter the transmembrane helix, perhaps increasing exposure to metalloproteases at caveolae. Furthermore, high levels of soluble EPCR have been reported to be associated with an increased prevalence of thrombosis although these clinical consequences have not been uniformly verified [14, 30]. In the study reported herein, the high-shedding G/G genotype of EPCR was more prevalent in SLE patients than controls. However, there were individuals with elevated soluble EPCR who did not carry the G allele. Recent investigations of the complex and polygenic inheritance patterns in SLE have shown an association at 20q11 [31], the same locus that contains the EPCR gene. Further study is needed to evaluate the relationship of this SNP to the vasculopathy of SLE in patient subgroups.

In addition to soluble EPCR, this study also evaluated circulating endothelial cells as another read out of endothelial dysfunction. All circulating endothelial cells from controls had membrane-bound EPCR. The detection of EPCR-negative circulating endothelial cells in SLE patients adds further support to the notion that shedding of EPCR is a component of endothelial priming in SLE. Unexpectedly, in contrast to the in vitro studies, no direct correlation between these two endothelial-specific markers was found (i.e., some patients had both a high soluble EPCR and a high percentage of membrane-bound EPCR-positive circulating endothelial cells). One possible explanation is that soluble EPCR may be derived from the microvasculature (e.g., renal) while circulating endothelial cells originate from the macrovasculature (e.g., arteries). An alternative explanation may be that SLE patients have both increased synthesis of EPCR and increased shedding.

CONCLUSION

In sum, the vascular dysfunction characteristic of SLE is associated with a dramatically altered distribution of EPCR, both soluble and membrane-bound forms. Circulating endothelial cells absent membrane-bound EPCR, increased soluble EPCR levels, and EPCR gene polymorphisms may predict and/or reflect vasculopathy and renal injury in SLE.

ACKNOWLEDGMENTS

This work was supported in part by research grants from the SLE Foundation, Inc., New York Chapter (R. Clancy), the Lupus Research Institute (R. Clancy), and the New Jersey Chapter of the Lupus Foundation of America, Inc. (R. Clancy). We thank Dr. Peter Krsko and Dr. Matt Libera of the Stevens Institute of Technology, who analyzed circulating endothelial cells using the electron microscopy technique. This work was supported in part by the SLE Foundation, Inc., New York Chapter and the Lupus Research Institute.

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