Persistent Increase in Plasma Thrombomodulin in Patients with a History of Lupus Nephritis: Endothelial Cell Activation Markers

RINIE FRIJNS, ROB FIJNHEER, ANJA SCHIEL, RICHARD DONDERS, JAN SIXMA, and RONALD DERKSEN

ABSTRACT. ***Objective.*** To investigate the presence of continuing endothelial cell activation in patients with systemic lupus erythematosus (SLE) and its relationship with lupus nephritis.

Methods. We measured plasma concentrations of soluble thrombomodulin (sTM), vascular cellular adhesion molecule-1 (sVCAM-1), von Willebrand factor (vWf), sP-selectin, and ED1-fibronectin in 75 SLE patients with a median SLE disease activity index (SLEDAI) of 4. Forty patients with a history of lupus nephritis, confirmed by renal biopsy in 33, were compared with 35 patients without lupus nephritis and 25 controls. For subgroup analysis in patients with clinically stable remission we excluded patients with a SLEDAI > 6 or with evidence of renal disease activity.

Results. In the total SLE patient group sTM, sVCAM-1, vWf, and sP-selectin were significantly elevated compared with controls. In patients with a history of lupus nephritis plasma levels of sTM and vWf were significantly increased compared with SLE patients without nephritis. After adjustment for significantly associated variables, especially creatinine clearance and age, in a multivariate linear regression analysis, sTM remained significantly elevated in patients with a history of lupus nephritis (difference 28.9 ng/ml, 95% CI 11.5–46.4). In the subgroup analysis of 57 patients, the results remained unchanged.

Conclusion. The increase of sVCAM-1, sP-selectin, sTM, and vWf reflects a state of persistent endothelial cell activation. Multivariate regression analysis shows that the elevated sTM levels are strongly associated with a history of lupus nephritis, independent of creatinine clearance or disease activity, suggesting endothelial cell activation specifically localized in the kidneys. (J Rheumatol 2001;28:514–9)

Key Indexing Terms:
ENDOTHELIAL CELL LUPUS NEPHRITIS THROMBOMODULIN

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease that is complicated by glomerulonephritis in more than 50% of patients. Clinical signs of renal disease vary from mild proteinuria to a rapidly progressive glomerulonephritis with endstage renal failure requiring hemodialysis or kidney transplantation. Histological findings of kidney biopsies are graded from I to VI according to the WHO classification. Focal and diffuse proliferative glomerulonephritis (class III and IV) are the most frequent[1].

From the Department of Neurology, Department of Haematology, Thrombosis and Haemostasis Laboratory, and Department of Rheumatology and Clinical Immunology, University Medical Center Utrecht, Utrecht, The Netherlands.

C.J.M. Frijns, MD, Neurologist; R.C.J.M. Donders, MD, PhD, Neurologist, Department of Neurology; R. Fijnheer, MD, PhD, Haematologist; J.J. Sixma, MD, PhD, Professor of Haematology, Department of Haematology; A. Schiel, Biologist, Thrombosis and Haemostasis Laboratory; R.H.W.M. Derksen, MD, PhD, Internist and Clinical Immunologist, Department of Rheumatology and Clinical Immunology.

Address reprint requests to Dr. C.J.M. Frijns, Department of Neurology, University Medical Center Utrecht, PO Box 85500, 3508 GA Utrecht, The Netherlands. E-mail: C.J.M.Frijns@neuro.azu.nl

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Immunologically mediated vascular endothelial cell activation is considered a pathogenetic factor in the disruption of normal organ function in SLE. To investigate continuing endothelial cell activation in SLE and its relationship with lupus nephritis, we measured the plasma concentrations of markers of endothelial cell activation in 75 outpatients with SLE and in 25 healthy controls. We selected 5 potential markers: soluble vascular cellular adhesion molecule (sVCAM-1), thrombomodulin (sTM), von Willebrand factor (vWf), sP-selectin, and ED1-fibronectin (ED1-fn).

VCAM-1 was selected because it proved to be a reliable marker and predictor of disease activity in SLE[2,3]. VCAM-1 is an inflammatory adhesion molecule belonging to the immunoglobulin gene superfamily of adhesion molecules that is involved in leukocyte adhesion and is expressed on endothelial cells after stimulation. Thrombomodulin is a glycoprotein located on the endothelial cell surface. Recently, plasma TM has also been shown to be associated with disease activity[4–6]. Von Willebrand factor is an adhesive glycoprotein stored in endothelial Weibel-Palade bodies and platelet alpha-granules. Von Willebrand factor was selected since it is regarded as the best circulating marker of endothelial cell activation because of its sensi-

tivity, its long plasma half-life, and its specificity for endothelial cells. Almost all circulating vWf originates from endothelium, whereas the amount from platelets is negligible[7,8]. Increased circulating vWf is found in inflammatory and atherosclerotic diseases[7–9]. Elevated concentrations have also been reported in SLE, predominantly in active disease[10–12]. P-selectin belongs to the selectin family of adhesion molecules and is a membrane protein colocalized with vWf on the Weibel-Palade bodies and the alpha-granules. Increased concentrations of sP-selectin are reported in disorders accompanied by endothelial cell activation[9,13–15]. Moderately elevated sP-selectin levels were reported in a small study in 21 patients with SLE[16]. At present, it is not clear which amount of sP-selectin is derived from platelets and which from endothelial cells. Plasma fibronectins are adhesive glycoproteins that are mainly produced by hepatocytes. Cellular ED1-fibronectin is a variant containing an extra domain (ED1) produced by alternative mRNA splicing. ED1-fibronectin is exclusively secreted by endothelial cells and fibroblasts[17]. Its concentrations are elevated in several disorders in which endothelial cell activation is present[13,18].

MATERIALS AND METHODS

Patients. SLE patients were selected from a cohort of 175 consecutive patients attending the outpatient lupus clinic of the Department of Rheumatology and Clinical Immunology. After they consented to participate, all patients in the cohort were interviewed using a standardized questionnaire, and had a physical examination. For additional data clinical charts were reviewed. All patients fulfilled at least 4 criteria of the American College of Rheumatology (revised ACR criteria) for the diagnosis of SLE[19]. Lupus nephritis was diagnosed if patients fulfilled the ACR criteria for renal involvement, i.e., persistent proteinuria > 0.5 g/24 h or cellular casts in the absence of infection[19]. A selection of 80 patients was made by the first author, who was unaware of relevant clinical details of the patients. Five of these 80 patients were excluded: 4 because of renal dialysis and one with increasing serum creatinine and decreased creatinine clearance who did not fulfil the ACR criteria for lupus nephritis. Disease activity was measured by the SLE Disease Activity Index (SLEDAI)[20]. Theoretically the maximum score of the SLEDAI is 105; in clinical practice, scores above 48 are very unusual. Hypertension was defined as systolic blood pressure ≥ 160 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg. Control patients were recruited from the outpatient clinic of the Department of Internal Medicine of University Medical Center Utrecht. They visited the clinic because of a diversity of problems unrelated to vascular or autoimmune diseases, such as unexplained gastrointestinal complaints, fatigue, and myalgia. In the controls the median age was 47 years (range 18–77), 68% were female, and none had hypertension. Serum creatinine ranged from 50 to 100 µmol/l (median 73).

Characteristics of the patients with and without lupus nephritis and controls are shown in Table 1. Most patients were in clinically stable remission, whereas some had mild disease activity. In the entire patient population the median SLEDAI score was 4 (range 0–12). The majority of the SLEDAI scores were determined by elevated anti-dsDNA antibodies and by complement levels below the normal limit of our laboratory, each counting for 2 points on the SLEDAI and present in 46 patients. Lupus headache, defined as persistent (migrainous) headache unresponsive to narcotic analgesia at the time of the visit or in the preceding 10 days, scores 8 points on the SLEDAI and accounted for the highest scores in our patient population (n = 4). In 2 patients the SLEDAI score included renal items (4 points each): one had a recent increase in proteinuria of > 0.5 g/24 h and another had urinary cellular casts. Erythrocytes or > 5 leukocytes per high power field were not found.

Forty patients met the ACR criteria for lupus nephritis; median time since diagnosis of nephritis was 4.8 years (range 0.2–20). In 33 patients a renal biopsy had been performed. Renal biopsy was not done in 7 patients because of coagulation disorders in 4, mild proteinuria in 2, and pregnancy in one patient. According to the WHO classification for lupus nephritis, biopsy specimens were classified as class II (n = 6), class III (n = 5), class IV (n = 20), class V (n = 1), and class VI (n = 1). At the time of examination, creatinine clearances, calculated according to the formula of Cockcroft and Gault[21], ranged from 19 to 159 ml/min (median 82). Serum creatinine concentrations ranged from 52 to 342 µmol/l (median 86.5); it was elevated in 10 patients (normal values 50–120 µmol/l). Proteinuria was present in 28 patients (median 0.4 g/l, range 0.1–5.7). Twenty-eight patients were treated with prednisone, 12 patients used azathioprine, and 3 were still in the course of treatment with pulse intravenous cyclophosphamide every 3 months that had been started 12 to 21 months before the date of examination.

Table 1. Patient characteristics.

	Nephritis +, n = 40	Nephritis –, n = 35	Controls, n = 25
Female (%)	35 (87.5)	32 (91.4)	17 (68)
Age, yrs	35.5 (22–69)	36.0 (20–60)	47.0 (18–77)
Disease duration of SLE, yrs	10 (1–38)	10 (1–26)	—
Hypertension (%)*	22 (55)	9 (25.7)	0
SLEDAI	4 (0–12)	3 (0–12)	—
Serum creatinine, µmol/l	86.5 (52–342)	71 (47–120)	73 (50–100)
Estimated creatinine clearance, ml/min†	82 (19–159)	94 (55–138)	—
History of urinary cell casts (%)	26 (65)	0	—
History of proteinuria (%)	40 (100)	0	—
Renal biopsy (%)	33 (82.5)	0	—
Current immunosuppressive drugs (%)	28 (70)	11 (33.3)	—
Prednisone	28 (70)	10 (28.6)	
Azathioprine	12 (30)	4 (11.4)	
Methotrexate	1 (2.5)	0	
Cyclophosphamide	3 (7.5)	0	

Values are expressed as numbers (%) or as median (range). *Systolic ≥ 160 and/or diastolic ≥ 90 mm Hg. †Estimated creatinine clearance according to Cockcroft and Gault[21].

In the 35 patients with no history of nephritis, estimated creatinine clearances ranged from 55 to 138 ml/min (median 94). Creatinine clearance was decreased (55 mm/min) in one patient with a renal infarction 15 years earlier who did not develop lupus nephritis during a followup period of more than 5 years after inclusion in the study. Serum creatinine concentrations ranged from 47 to 120 µmol/l (median 71). None of the 36 patients had proteinuria or abnormal urine sediments. Ten patients were treated with prednisone and 4 with azathioprine.

For a separate analysis of markers in a subgroup of patients in whom the disease could be considered in stable remission, we excluded 18 patients. Reasons for exclusion were a SLEDAI > 6 (n = 7), and/or possible renal disease activity (n = 16) according to any of the following criteria: presence of urinary casts at the time of inclusion in the study; increase of serum creatinine concentration of > 30% or increase in proteinuria of > 0.5 g/24 h in the previous 3 months; renal biopsy, or institution of therapy with prednisone or azathioprine, or increase of dosage, in the past 12 months; treatment with cyclophosphamide at the time of inclusion in the study.

Blood sampling and laboratory investigations. Blood was sampled in citrate anticoagulant (1:10 in 3.1% citrate) and centrifuged immediately at 2000 g for 15 min at 4°C. The supernatant was removed and centrifuged a second time. Plasma samples were stored at –70°C. Commercial ELISA kits were used for measurement of sVCAM-1 (R&D Systems, Abingdon, UK) and sTM (Diagnostica Stago, Paris, France). sP-selectin, vWf, and ED1-fibronectin were measured with ELISA developed at our Research Laboratory of Thrombosis and Haemostasis. Briefly, microtiter plates were coated overnight with specific monoclonal antibodies (Mab) at 4°C and then blocked. Each plate contained 8 concentrations of a standard. This was either recombinant P-selectin (R&D Systems), pooled human serum, or ED1-fn purified from cultured lung fibroblasts. For the sP-selectin ELISA we used Mab 2.15 to capture and biotinylated Mab 1.18 for detection, both recognizing different epitopes[13,22]. For the vWf ELISA we used anti-human vWf (Dako) to capture and peroxidase coupled anti-human vWf (Dako) for detection. ED1-fn was captured with IgM Mab 3E2 raised against fibronectin antigen released by cultures of human breast cancer cell lines[23,24] (Sigma, St. Louis, MO, USA) and detected with biotinylated anti-human fibronectin $F(ab)_2$ fragments. Biotinylated second Mab and $F(ab)_2$ were incubated with streptavidin/horseradish peroxidase. Staining was done with *o*-phenylenediamine dihydrochloride. Optical densities were read at 490 nm with the V_{max} kinetic microplate reader (Molecular Devices, Corp. Menlo Park, CA, USA). vWf was expressed as percentage of the value in pooled plasma of 40 healthy donors (10 µg/ml).

Statistical evaluation. Data entry and calculations were performed with the SPSS statistical software package. Results are presented as mean values ± SD. Differences between groups were calculated with the Mann-Whitney U test. Associations between markers and relevant demographic and clinical data were analyzed with univariate and multivariate regression. Associations were expressed with 95% confidence limits. A p value < 0.05 for differences and associations was considered statistically significant.

RESULTS

Concentrations of endothelial cell activation markers. Overall, significant differences between SLE patients and controls were found for sTM, sVCAM-1, vWf, and sP-selectin, but not for ED1-fibronectin. Considering the mean value + 2 SD in controls the upper limit of normal, at least one marker was elevated in 46 patients (61.3%). As in the entire SLE patient group, all markers except ED1-fn were significantly elevated in patients with lupus nephritis compared with controls, whereas only vWf and sP-selectin were significantly increased in patients without lupus nephritis compared with controls (Table 2, Figure 1). Comparing patients with lupus nephritis to patients without lupus nephritis, there was a remarkable difference in sTM concentrations ($p < 0.001$), whereas concentrations in patients without lupus nephritis were the same as in controls (Table 2, Figure 1). vWf was also significantly elevated in patients with lupus nephritis compared to those without ($p < 0.05$).

Associations between markers and relevant clinical and demographic variables. Univariate linear regression analysis. In the SLE patients we found significant positive associations between sTM and age ($p = 0.008$), lupus nephritis ($p < 0.001$), hypertension ($p = 0.004$), presence of proteinuria ($p = 0.001$), and prednisone treatment ($p = 0.024$), and a negative association with creatinine clearance ($p < 0.001$). sVCAM-1 was significantly associated with age ($p = 0.001$), lupus nephritis ($p = 0.017$), and disease duration ($p = 0.001$), and negatively with creatinine clearance ($p < 0.001$), and vWf with age ($p = 0.007$), lupus nephritis ($p = 0.003$) and prednisone treatment ($p = 0.024$). There were no statistically significant associations between sP-selectin or ED1-fibronectin with any of these variables. None of the markers was significantly associated with sex, with the SLEDAI, or with a history of arterial and/or venous thrombotic disease.

Multivariate regression analysis. Significantly associated variables identified in the univariate regression analysis were entered into a multivariate regression model. sTM was independently associated with lupus nephritis ($p = 0.002$)

Table 2. Mean concentrations ± SD of circulating endothelial cell markers in patients with a history of lupus nephritis, in SLE patients without nephritis, and in healthy controls.

	Nephritis+, n = 40	Nephritis–, n = 35	Controls, n = 25
sThrombomodulin, ng/ml	66.6 ± 41.9†a ‡a	29.1 ± 10.1	29.1 ± 11.5
sVCAM-1, ng/ml	679.3 ± 254.4†b	560.4 ± 136.3	510.7 ± 135.6
vWf, %*	184.3 ± 65.7†a ‡c	153.9 ± 60.8†c	115.0 ± 52.4
sP-selectin, ng/ml	175.5 ± 64.8†a	166.9 ± 39.8†a	128.8 ± 41.0
ED1-fibronectin, µg/ml	1.90 ± 0.90	1.84 ± 1.26	1.69 ± 0.9

†p values compared with controls: †a$p < 0.001$, †b$p < 0.01$, †c$p < 0.05$.
‡p values compared with patients without history of lupus nephritis: a$p < 0.001$, b$p < 0.01$, c$p < 0.05$.
*Percentage of value in pooled plasma of 40 healthy donors with concentration of 10 µg/ml.

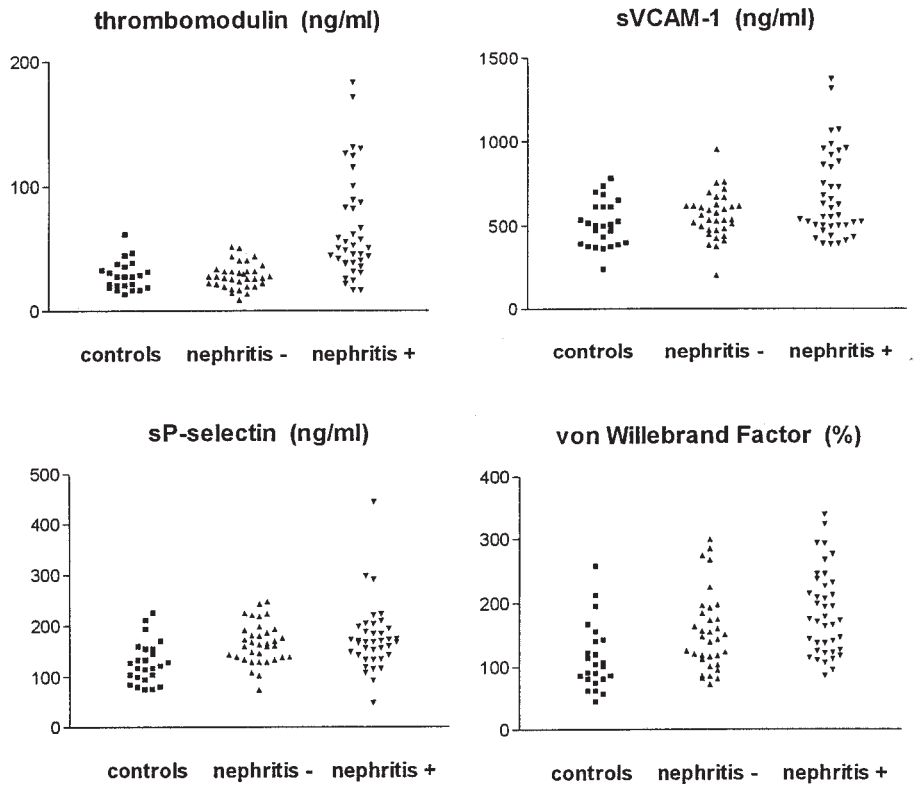

Figure 1. Comparison of plasma concentrations of thrombomodulin, sVCAM-1, sP-selectin, and von Willebrand factor in SLE patients with nephritis (nephritis+, n = 40), SLE patients without nephritis (nephritis–, n = 35), and controls (n = 25).

and creatinine clearance (p = 0.001) (Table 3). sVCAM-1 was also independently associated with lupus nephritis (p = 0.046) and with creatinine clearance (p = 0.042). Although age was still significantly associated with vWf (p = 0.004), the associations between vWf and prednisone treatment and lupus nephritis were lost (Table 3). After adjustment for age and serum creatinine the statistical differences in sTM, vWf, and sVCAM-1 concentrations between SLE patients and controls remained unchanged.

Subgroup analysis in 57 patients with clinically stable remission. For this analysis 18 patients, 16 with and 2 without a history of lupus nephritis, were excluded according to the criteria described above. In the remaining patients, nonparametric between-group comparisons showed essentially the same statistical differences as in the entire study population of 75 patients. The multivariate regression analysis showed a strong association of sTM with history of lupus nephritis (p < 0.001; B = 41.3; 95% CI 21.5,

Table 3. Associations between markers and clinical variables: adjustment for significantly associated variables from the univariate analysis in a multivariate linear regression analysis.

	sTM			sVCAM-1			vWf		
	B*	(95% CI)	p	B	(95% CI)	p	B	(95% CI)	p
Lupus nephritis**	28.9	(11.5, 46.4)	0.002	91.5	(1.5, 181.5)	0.046	19.9	(–10.6, 50.4)	0.198
Creatinine clearance	–0.4	(–0.6, –0.2)	0.001	–1.6	(–3.2, –0.1)	0.042		—	
Age	0.6	(–0.1, 1.3)	0.115	3.8	(–1.5, 9.0)	0.155	2.0	(0.6, 3.4)	0.004
Disease duration		—		6.5	(–0.4, 13.3)	0.064		—	
Hypertension**	8.9	(–5.0, 22.8)	0.206		—			—	
Prednisone treatment**	–0.2	(–15.3, 14.9)	0.981		—		25.1	(–5.4, 55.5)	0.105
Presence of proteinuria**	–0.1	(–18.4, 18.3)	0.992		—			—	

*The regression coefficients B should be interpreted as the increase in concentration for each unit increase of the variable studied, e.g., for one year increase of age, thrombomodulin level would increase by 0.6 ng/ml. For the dichotomized variables the coefficient represents the difference in concentration between presence and absence of the variable.

**In the case of dichotomized variables, presence of the variable = 1 and absence = 0.

61.1) and with creatinine clearance (p = 0.031; B = –0.3, 95% CI –0.60, –0.03). There was still a significant association of sVCAM-1 with disease duration (p = 0.048), but not with creatinine clearance or age, and a trend towards a positive association with lupus nephritis (p = 0.088). The association of vWf with age remained unchanged.

DISCUSSION

We found elevated concentrations of 4 out of the 5 investigated endothelial cell activation markers in patients with SLE compared with controls. In patients with a history of lupus nephritis, unadjusted values of sTM and vWf were significantly increased compared with patients without lupus nephritis. After selection of a subgroup of patients with clinically stable remission, concentrations remained elevated, suggesting a state of permanent activation of endothelial cells in SLE.

Since hypertension has been reported to influence endothelial cell function[14,25], its presence in a large proportion of patients with SLE could confound elevated plasma levels of endothelial cell markers attributed to SLE itself. Treatment with prednisone may be another confounder. However, we could not observe an independent influence of hypertension or prednisone treatment on circulating levels of the investigated endothelial cell activation markers.

Little is known about plasma half-life and clearance of these endothelial molecules. Statistically significant correlations between sTM and serum creatinine have been reported in patients with SLE during disease exacerbations[4,6], and even in healthy controls[6,26]. We also found an association between sTM and creatinine clearance in SLE. After adjustment for associated variables in a multivariate analysis, both the association of sTM with creatinine clearance and the difference in sTM concentrations between patients with and without lupus nephritis remained statistically significant. This was also the case in the subgroup analysis of patients with stable remission. This finding suggests that sTM, which consists of molecules with a relatively low molecular weight (28 to 80 kDa)[27], is renally cleared. On the other hand, our data suggest that decreased renal clearance is not enough to explain the increased sTM concentration in patients with a history of lupus nephritis, and that these patients may have increased endothelial synthesis and expression of TM, in particular in the kidneys. The reported increased expression of TM along the capillary wall of the glomeruli in lupus nephritis, in contrast to several other renal diseases, supports this explanation[28].

The discrepancy between the other markers and sTM in patients with lupus nephritis compared with patients without lupus nephritis may be explained by endothelial cell heterogeneity[29]. This would imply that persisting endothelial cell activation in SLE leads to elevation of different endothelial cell markers depending on the involved tissue or organ.

sVCAM-1 is reported to be especially elevated at times of disease exacerbation in patients with renal involvement[2]. We also found a statistically significant difference in concentrations in patients with and without lupus nephritis. However, the significant independent associations of sVCAM-1 with creatinine clearance and lupus nephritis in the entire study population disappeared after selection of a subgroup of patients with clinically stable remission. Molecular weights of the soluble form of 50 to 90 kDa have been reported[30]. We suggest that sVCAM-1 may also, at least partly, be renally cleared.

The rise in vWf and sP-selectin concentrations in patients with SLE compared with controls supports the hypothesis of a persistent increase in endothelial cell activation. We found no relationship between these molecules and creatinine clearance, which is to be expected in view of their high molecular weight. Although in the unadjusted comparisons vWf was increased in patients with a history of lupus nephritis compared with those without lupus nephritis, this difference disappeared in the multivariate analysis. We conclude that history of lupus nephritis does not influence the plasma concentrations of vWf and sP-selectin, suggesting that it is not the renal component of SLE that is responsible for their increased release.

A recent study shows significantly increased concentrations of ED1-fibronectin in 6 out of 16 SLE patients with various signs of active disease[31]. However, we did not observe an increase of ED1-fibronectin concentration in the SLE patient group as a whole, nor in the subgroup of patients with lupus nephritis, although increased deposition of ED1-fibronectin has been described in the mesangial and interstitial matrix in lupus nephritis[32]. Most of the ED1-fibronectin in these compartments may have accumulated during disease exacerbations. ED1-fn synthesis in the endothelial cells may be too low in mildly active SLE to lead to a measurable increase in plasma. On the other hand, if endothelial cell polarity is intact, increased quantities of ED1-fn may be strictly secreted into the matrix at the abluminal side and not into the circulation.

Our results cannot distinguish with certainty between endothelial cell activation and damage. However, elevated levels of sTM, sVCAM-1, vWf, and sP-selectin indicate that endothelial cells are not damaged to such an extent that they have become incapable of protein synthesis. Increased expression and release of these molecules also suggest that endothelial cells still possess the capacity to be stimulated by agents such as interleukins or thrombin. Although a mildly damaged cell may perhaps retain these qualities, we prefer the use of endothelial cell activation instead of damage. Moreover, it cannot be excluded that activated but normal endothelial cells may coexist with damaged cells in the same patient.

We suggest that elevated plasma concentrations of sTM, sVCAM-1, vWf, and sP-selectin reflect persisting endothelial cell activation in SLE. Elevated thrombomodulin

concentrations are associated with a history of lupus nephritis independent of disease activity or renal clearance, suggesting endothelial cell activation particularly in the kidneys. Clinical significance of these findings may be confirmed in longitudinal prospective studies.

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