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CORRELATION BETWEEN INCREASED NITRIC OXIDE PRODUCTION AND MARKERS OF ENDOTHELIAL ACTIVATION IN SYSTEMIC SCLEROSIS

Findings With the Soluble Adhesion Molecules E-Selectin, Intercellular Adhesion Molecule 1, and Vascular Cell Adhesion Molecule 1

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Objective. **To determine the relationship between vascular function and the inflammatory response in systemic sclerosis (SSc), and to investigate whether production of endothelial-derived nitric oxide (NO) is disturbed in this disease.**

Methods. **We measured plasma nitrate, urinary excretion of both nitrate and cGMP, and soluble adhesion molecules of endothelial origin in patients with SSc and in age- and sex-matched controls and compared these levels between groups. Additionally, we performed correlation analysis to determine how these variables were related to one another. Plasma nitrate and 24-hour-urinary excretion of nitrate in patients and controls were measured after a 72-hour nitrate-free-diet, using a gas chromatography/mass spectrometric method. Soluble adhesion molecules intercellular adhesion molecule 1 (sICAM-1), vascular cell adhesion molecule 1 (sVCAM-1), and E-selectin and cytokines were measured by enzyme-linked immunosorbent assay. The expression of E-selectin was further investigated in skin biopsy specimens by immunoperoxidase staining, and the presence of inducible NO synthase by immunoblotting.**

Results. **Plasma nitrate and 24-hour-urinary-excretion of cGMP were significantly elevated in patients compared with controls, while 24-hour-urinary-excretion of nitrate tended to be elevated in SSc patients. Levels of sICAM-1, sVCAM-1, and sE-selectin were significantly elevated in the patients. Levels of plasma nitrate in the patients correlated significantly with levels of sVCAM-1 ($P = 0.020$) and sE-selectin ($P = 0.018$) and approached a significant correlation with sICAM-1 ($P = 0.055$), suggesting that activated endothelial cells may produce plasma nitrate.**

Conclusion. **NO synthesis is elevated in SSc patients, and the activated endothelial cell is a likely site of its production.**

Systemic sclerosis (SSc; scleroderma) is a generalized autoimmune inflammatory disorder of connective tissue, characterized by microvascular and immunologic abnormalities and increased fibroblast activity. The disease leads to fibrosis in skin and internal organs (1). Histopathologic findings include intimal and medial concentric hypertrophy with lumen reduction throughout the arterial tree (2). The vasculature seems to play a major role in the pathogenesis of SSc. For example, the prognosis and outcome of the disease are dependent on the extent and severity of the vascular lesions (3,4), and vascular involvement in the heart, lungs, and kidneys leads to increased morbidity and mortality (5–9).

We have previously shown that left ventricular hypertrophy, diastolic dysfunction (10), and cold-inducible perfusion defects of the myocardium are common in SSc (11), suggesting myocardial vascular engagement. We and others have reported elevated serum

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endothelin levels in SSc (12,13), providing further evidence that the vasculature plays a role in this disease.

Attention has been increasingly focused on the endothelial cell as an important modulator of the inflammatory response as well as of vascular tone. A variety of markers of endothelial activation are elevated in SSc, such as von Willebrand factor, tissue plasminogen activator (14), soluble E-selectin (sE-selectin), soluble vascular cell adhesion molecule 1 (sVCAM-1), soluble intercellular adhesion molecule 1 (sICAM-1), and endothelin (12,13,15). It has been proposed that the vasodilator substance nitric oxide (NO), generated via inducible NO synthase (iNOS), plays a major role in the pathogenesis of autoimmune systemic rheumatic disease (16). However, studies on the production and role of NO in SSc have yielded conflicting results (17,18). An early study showed that NO levels were decreased in SSc (17), a notion that is supported by the demonstrated downregulation of endothelial NOS (eNOS) in this disease (19). However, elevated levels of plasma nitrate and enhanced staining for iNOS in endothelial cells, infiltrating monocytes, and fibroblasts in skin biopsy specimens from SSc patients have been demonstrated recently (18).

In view of the likely role of the vasculature in the pathophysiology of SSc and the conflicting literature pertaining to the role of NO in SSc, we investigated circulating factors related to endothelial cell activation and cytokines modulating the inflammatory response, as well as mediators of vascular tone (i.e., NO), in SSc. In addition, skin biopsy samples were obtained for immunohistochemical evaluation of local endothelial activation.

PATIENTS AND METHODS

Patients and controls. Twenty-seven consecutive patients (23 women and 4 men) with SSc according to the criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) (20) were studied. Twenty-three of the patients (21 women and 2 men) had limited cutaneous SSc (lcSSc) and 4 (2 women and 2 men) had diffuse cutaneous SSc (dcSSc) (21). Four of the SSc patients (all female) fulfilled the Alarcon-Segovia and Cardiel criteria for mixed connective tissue disease (MCTD) (22). Twenty-seven age- and sex-matched healthy subjects randomly selected from within the age cohort in the population register of the county of Västerbotten, Sweden served as controls.

At the time of investigation, 9 patients with lcSSc had anticentromere antibodies, 2 with lcSSc and 1 with dcSSc had anti–Scl-70 antibodies, and all 4 patients who fulfilled the criteria for MCTD had high-titer RNP antibodies. The mean ± SD disease duration from the time of diagnosis to study entry was 12.7 ± 9.9 years (range 1–38 years). All patients except 1 had Raynaud's phenomenon (23). Eight of these patients were severely affected, with digital pitting scars or ulcers, and 2 had had fingers amputated. Skin score was assessed according to the modified Rodnan model C with 8 unilateral sites and a maximum of 16 points (24), which resulted in a mean ± SD score of 2.3 ± 1.9. Esophageal dysmotility (diagnosed by radiographic examination) was found in 70% of the patients. Seven patients showed decreased (more than 2 SD below the expected value) single-breath diffusing capacity for carbon monoxide (DLCO). Nine patients were receiving corticosteroid treatment. Seven were treated with angiotensin-converting enzyme (ACE) inhibitors. Two patients with dcSSc had had renal crisis, with partly restored renal function (creatinine clearance 43 ml/minute and 23 ml/minute, respectively). Seven of the patients and 5 of the controls were current smokers.

Determination of nitrate levels in plasma and urine. Venous blood samples were obtained from patients and controls after they had been on a nitrate-free diet for 72 hours. Urine was collected during 24 hours prior to the study visit. Nitrate was measured with a stable isotope ($^{15}NaNO_3$) dilution assay utilizing positive ion/chemical ionization gas chromatography/mass spectrometry, after endogenous and labeled nitrate in the samples had been converted to nitrobenzene as described in detail elsewhere (25). This method gives a reliable measure of nitrate levels as an estimate of NO levels.

Determination of cGMP levels in urine. NO released from the endothelium in response to vasodilator stimuli diffuses to the adjacent smooth muscle cell. In the smooth muscle cell, NO activates guanylate cyclase and increases cGMP, which causes relaxation. Therefore, the downstream second messenger element to the NO stimulation, cGMP, was measured in urine by a radioimmunoassay using a commercial kit (New England Nuclear, Boston, MA).

Cytokines and adhesion molecules. Aliquots of sera were frozen at −80°C until assayed. Interleukin-1β (IL-1β), IL-6, IL-1 receptor antagonist (IL-1Ra), IL-10, tumor necrosis factor α (TNFα), sICAM-1, sVCAM-1, sE-selectin, and transforming growth factor β1 (TGFβ1) were each measured with specific enzyme-linked immunosorbent assays (R&D Systems Europe, Oxon, UK) according to the manufacturer's protocol. In each assay the appropriate recombinant human cytokine was used to generate the standard curve. Sensitivities of the various assays were as follows: IL-1β 1.0 pg/ml, IL-6 0.70 pg/ml, IL-1Ra 14 pg/ml, IL-10 1.5 pg/ml, TNFα 4.4 pg/ml, sICAM-1 7 ng/ml, sVCAM-1 <2 ng/ml, sE-selectin <0.1 ng/ml, TGFβ1 7 pg/ml. Measurements below detectable levels were used as the lower cutoff limit of the assay according to the instructions of the manufacturer. The value recorded was the mean of 2 measurements.

E-selectin expression. Skin biopsy specimens from 3 patients and 2 age- and sex-matched controls were fixed in 4% paraformaldehyde (Merck, Darmstadt, Germany) in 0.1*M* phosphate buffer, pH 7.3, for 4 hours at 4°C. After washing, the tissue was snap-frozen in liquid nitrogen and cut in 5 μm–thick sections with a cryostat microtome. Endogenous peroxidase activity was blocked with 0.003% hydrogen peroxide in 0.02*M* phosphate buffered saline (PBS) at 37°C for 30 minutes, followed by 3 15-minute washes in 0.02*M* PBS. Appropriate concentrations of anti–E-selectin monoclonal antibody (mAb) (clone 1.2B6; Ancell, Bayport, MN), anti-CD45 mAb (clones 2B11 and PD7/26; Dako, Glostrup, Denmark), and isotype-

matched irrelevant mAb (clone DAK-G01; Dako) as a negative control were applied to the sections and incubated for 2 hours at room temperature. After washing, incubation with a peroxidase-conjugated rabbit anti-mouse Ig (Dako) for 2 hours at room temperature was performed. The sections were then washed 3 times, developed for 10 minutes with a substrate solution containing 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) and 0.03% hydrogen peroxide in 0.05*M* Tris HCl, and finally counterstained with Gill's hematoxylin (Vector, Burlingame, CA) according to the instructions of the manufacturer.

NO synthase determination. Biopsy specimens for Western blotting were homogenized using an Ultra-Turrax T8 (IKA Labortechnik, Staufen, Germany) in 5 volumes of ice-cold homogenization buffer containing 25 mmoles/liter HEPES, pH 7.4, 0.1 mmole/liter EDTA, and a mixture of protease inhibitors (0.01 mg/ml phenylmethylsulfonyl fluoride, 0.01 mg/ml trypsin inhibitor, 0.01 mg/ml leupeptin, 0.01 mg/ml antipain, 0.01 mg/ml chymostatin, 0.01 mg/ml pepstatin; Sigma). After centrifugation at 10,000*g* for 45 minutes, the protein concentration of the lysate was determined using BCA protein assay reagent (Pierce, Rockford, IL). The cell suspension was heated to 70°C for 10 minutes in a reducing sample buffer. After separation of the homogenates with the NU Page Electrophoresis system in Tris-Acetate Gels (Novex, San Diego, CA), proteins were electroblotted onto polyvinylidene difluoride membranes (26). The membranes were incubated with 0.2% casein for 30 minutes at room temperature to block nonspecific binding. They were then incubated with mAb against iNOS and eNOS (Transduction Laboratories, Lexington, KY) overnight at 4°C. Appropriate secondary antibodies conjugated to alkaline phosphatase were used for visualization of the immunoreactive proteins by a chemiluminescence method (Western light plus; Tropix, Bedford, MA). The chemiluminescence reaction was detected using hyperfilm (ECL; Amersham, Pharmacia Biotech, Buckinghamshire, UK).

Statistical analysis. Wilcoxon's signed rank test was used for testing differences between patients and matched controls for continuous data. Differences between 2 independent groups were analyzed with the Mann-Whitney nonparametric test for continuous data and by chi-square test for categorical data. Spearman's rank correlation was used for testing correlations between variables. The calculations were performed using Stat View 4.5 (Brain Power, Calabasas, CA). Factor analysis was performed by SPSS with Quartimax with Kaiser normalization as a rotation method. All *P* values refer to 2-sided tests; *P* values less than 0.05 were considered significant.

RESULTS

Clinical characteristics. Characteristics of the patients and controls are summarized in Table 1. For ethical reasons, medication was not withdrawn prior to study participation. Levels of von Willebrand factor and triglycerides were significantly elevated in the patient group. Elevated levels of triglycerides were found exclusively in patients receiving corticosteroid treatment.

Table 1. Characteristics of the patients with systemic sclerosis and the age- and sex-matched controls*

	Patients (n = 27)	Controls (n = 27)
Age, years	56.9 ± 14.9	56.9 ± 14.1
Height, cm	166.1 ± 8.6	166.0 ± 8.5
Weight, kg	67.2 ± 10.6	67.4 ± 13
Blood pressure (systolic), mm Hg	142.9 ± 26.0	135.4 ± 22.2
Blood pressure (diastolic), mm Hg	78.9 ± 13.3	79.6 ± 9.3
ESR, mm/hour	17.9 ± 17.1	14.3 ± 12.5
Leukocytes, $\times 10^9$/liter	5.5 ± 1.8	5.6 ± 1.7
Platelets, $\times 10^9$/liter	237.8 ± 80.1	250.9 ± 58.9
CRP, mg/liter	10.5 ± 1.7	10.2 ± 0.7
Orosomucoid, gm/liter	0.8 ± 0.3	0.6 ± 0.2
Creatinine clearance (per 1.73 m^2), ml/minute	84.0 ± 32.2	92.7 ± 36.1
Von Willebrand factor, units/ml	1.72 ± 0.16†	1.17 ± 0.09
Cholesterol, mmoles/liter	5.4 ± 1.2	5.2 ± 0.8
Triglycerides, mmoles/liter	1.6 ± 0.8‡	1.3 ± 0.9
HDL cholesterol, mmoles/liter	1.3 ± 0.4	1.4 ± 0.5
Lipoprotein(a), mg/liter	264.6 ± 249.7	211.8 ± 205.3

* Values are the mean ± SD. See ref. 45 for methods and reference values. ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; HDL = high-density lipoprotein.
† $P = 0.009$ versus controls.
‡ $P = 0.008$ versus controls.

Nitrate in plasma and urine and urinary cGMP. Plasma nitrate and urinary excretion of cGMP were significantly elevated in SSc patients compared with controls (median 25.9 μM versus 20.0 μM and 0.848 nmoles/mg creatinine versus 0.729 nmoles/mg creatinine, respectively) (Figures 1a and c). Levels of plasma nitrate and urinary cGMP excretion were not significantly correlated in patients ($P = 0.44$). Urinary excretion of nitrate was increased in patients compared with controls (median 0.529 μmoles/mg creatinine versus 0.419 μmoles/mg creatinine) (Figure 1b); however, the increase was not significant.

NO synthase expression. Skin biopsy specimens from 3 SSc patients and 1 control subject were analyzed for eNOS and iNOS by Western blotting (Figure 2). Staining for eNOS and iNOS was demonstrated in 2 patients. In the third patient and in the control, no staining was seen.

Adhesion molecules. Serum levels of adhesion molecules sICAM-1, sVCAM-1, and sE-selectin were all significantly increased in patients compared with controls (Figures 1d–f). Median levels of sICAM-1, sVCAM-1, and E-selectin, respectively, were 274.5 ng/ml versus 220.0 ng/ml, 693.0 ng/ml versus 572.0 ng/ml, and 59.3 ng/ml versus 45.6 ng/ml.

E-selectin expression on the endothelium of the subepithelial vessels in scleroderma skin samples. As illustrated in Figure 3, E-selectin was expressed on the

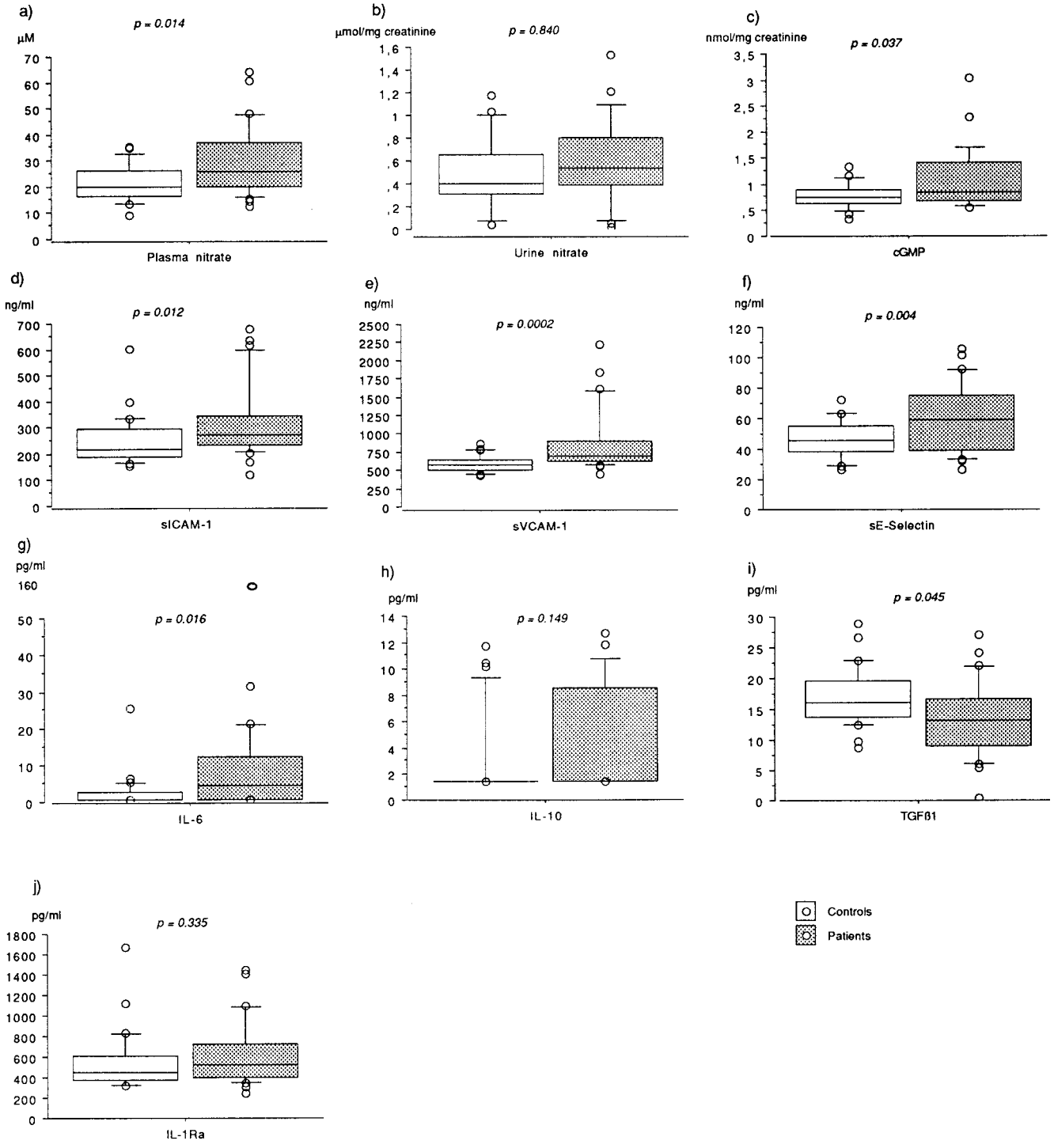

Figure 1. Box plots showing the levels of plasma nitrate (**a**), urine nitrate (**b**), urine cGMP (**c**), soluble intercellular adhesion molecule 1 (sICAM-1) (**d**), soluble vascular cell adhesion molecule 1 (sVCAM-1) (**e**), soluble E-selectin (**f**), interleukin-6 (IL-6) (**g**), IL-10 (**h**), transforming growth factor β1 (TGFβ1) (**i**), and IL-1 receptor antagonist (IL-1Ra) (**j**) in patients with systemic sclerosis (n = 27) and age- and sex-matched controls. Boxes show the 25th and 75th percentiles; horizontal lines in boxes show the medians; bars above and below boxes show the 10th and 90th percentiles; open circles show outlying values. *P* values were determined by Wilcoxon's signed rank test.

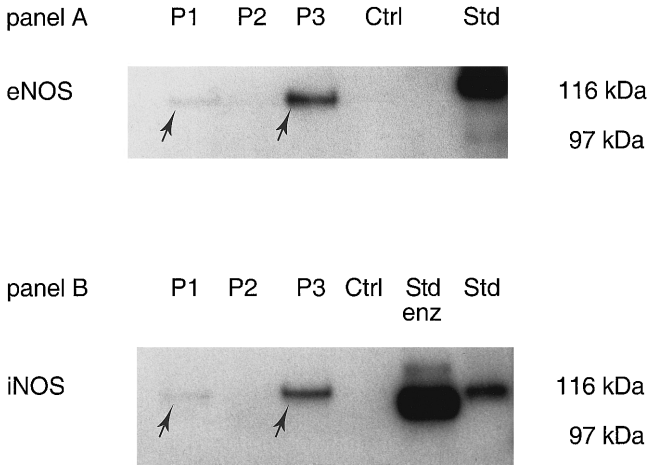

Figure 2. Western blotting of skin biopsy samples from 3 systemic sclerosis patients (P1, P2, and P3) and 1 healthy control subject (Ctrl). **Arrows** indicate bands of endothelial nitric oxide synthase (eNOS) and inducible NOS (iNOS). Std = standard; Std enz. = standard enzyme (a strongly positive reference). The 1 patient whose specimen showed marked staining for both eNOS and iNOS was a 49-year-old woman who had had diffuse sclerodermatous skin changes of the limbs and trunk (edematous stage) for 3 years; the biopsy specimen was obtained from her upper arm.

surface of the luminal endothelial cells from patients with SSc. In one of the stained samples, infiltrates of lymphoid cells were observed. Staining with mAb against common leukocyte antigen CD45 (Figure 3D) confirmed that these cells were leukocytes, indicating that the E-selectin expressed on the endothelium is one of the markers responsible for the extravasation of inflammatory cells through vessels to the dermis. In contrast, samples from 2 healthy donors showed no E-selectin staining and no leukocyte infiltration (results not shown). Control studies using irrelevant isotype-matched antibodies in the first mAb incubation confirmed the specificity of the immunoperoxidase staining (Figure 3C).

Cytokines, cytokine receptor antagonist, and growth factor. IL-6 was detectable in only 15 patients and 10 controls (median levels in the SSc group and the control group 3.9 pg/ml and 0.7 pg/ml, respectively; $P = 0.016$) (Figure 1g). Median levels of IL-1Ra were 529 pg/ml and 454 pg/ml in the patient and control groups, respectively (Figure 1j). Although there was not a significant difference between groups in the median IL-10 levels (Figure 1h), IL-10 was detected significantly more frequently in patient sera (11 of 27) than in control sera (3 of 27) ($\chi^2 = 6.17$, $P < 0.02$). TGFβ1 levels were significantly lower in the patients versus the controls (median 13.05 ng/ml and 16.2 ng/ml; $P = 0.045$) (Figure 1i). IL-1β and TNFα were not detectable in patients or controls.

Correlations between plasma nitrate and soluble adhesion molecule levels. Levels of sVCAM-1 and sE-selectin correlated significantly with levels of plasma nitrate in the patient group (Table 2). When the data from the control group and the patient group were combined, there was an even stronger correlation between sVCAM-1 or sE-selectin levels and the plasma nitrate level (Table 3). However, within the control group, there was no correlation between sVCAM-1 or sE-selectin levels and the plasma nitrate level. In the patient group, the correlation between sICAM-1 and plasma nitrate levels approached, but did not reach, significance ($r_s = 0.384$, $P = 0.055$). In the control group, these variables did correlate significantly (Figure 4A). When the data from the patient and control groups were

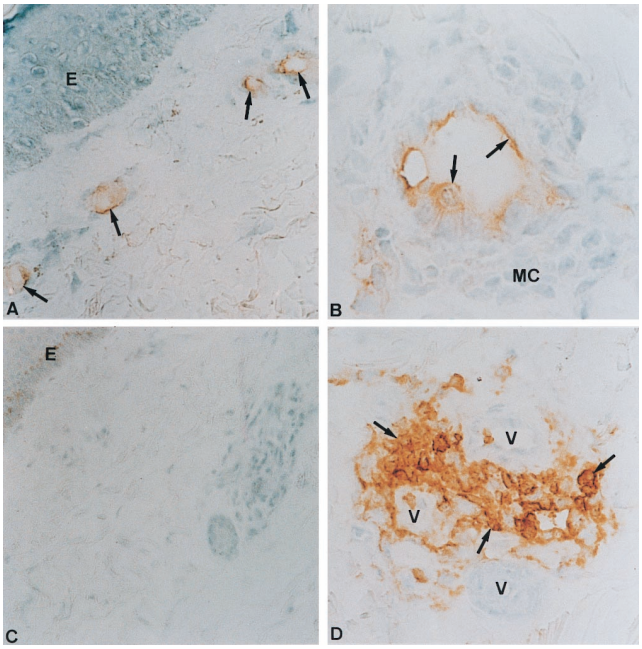

Figure 3. Immunoperoxidase staining for E-selectin (**A** and **B**) and CD45 antigen (**D**), and control staining (**C**). **A,** Skin specimen, showing 4 dermal capillaries (**arrows**), stained with monoclonal antibody (mAb) against E-selectin. **E** = epidermis. **B,** Capillary in the subepidermal portion of the dermis, showing E-selectin–positive staining of the endothelial surface facing the lumen of the vessels (**arrows**) and in close contact with the endothelial cells. **MC** = mononuclear cells. **C,** Staining with isotype-matched inappropriate mAb (negative control). **D,** Cross-section of skin from the same patient as in **B,** showing a group of blood vessels (**V**), surrounded by leukocytes, stained with mAb CD45 (**arrows**). The dermis from healthy donors remained unstained (not shown). (Original magnification × 20 in **A** and **C**; × 40 in **B** and **D.**)

Table 2. Spearman's rank correlation coefficients for correlations between levels of p-NO, u-NO, urine cGMP, soluble adhesion molecules (sICAM-1, sVCAM-1, and sE-selectin), and IL-6 in 27 patients with systemic sclerosis*

Variable	u-NO	cGMP	sICAM-1	sVCAM-1	sE-selectin	IL-6
p-NO	0.648†	0.047	0.384	0.465‡	0.472‡	0.207
u-NO		0.132	0.092	0.246	0.191	0.179
cGMP			0.130	0.056	−0.089	0.068
sICAM-1				0.554†	0.636†	0.360
sVCAM-1					0.623†	0.652§
sE-selectin						0.239

* Plasma nitrate (p-NO) and urine nitrate (u-NO) were used as indicators of nitric oxide. sICAM-1 = soluble intercellular adhesion molecule 1; sVCAM-1 = soluble vascular cell adhesion molecule 1; IL-6 = interleukin-6.
† $P < 0.01$.
‡ $P < 0.05$.
§ $P < 0.001$.

combined, the correlation between sICAM-1 and nitrate levels remained significant (Table 3 and Figure 4A). Figures 4B and C show correlations between levels of plasma nitrate and sVCAM-1 (Figure 4B) and sE-selectin (Figure 4C).

Correlations with laboratory and clinical data. Among the SSc patients, there were no significant correlations between levels of IL-6, IL-10, TGFβ1, adhesion molecules, plasma nitrate, urinary nitrate, or cGMP on the one hand and the acute-phase reactants erythrocyte sedimentation rate (ESR), orosomucoid, or haptoglobin on the other (data not shown). In controls, however, IL-6 levels correlated significantly with values for ESR ($r_s = 0.705$, $P < 0.002$), haptoglobin ($r_s = 0.594$, $P < 0.007$), and orosomucoid ($r_s = 0.424$, $P = 0.081$). There was no association between the degree of severity of Raynaud's phenomenon, skin score, disease duration, or DLCO and levels of plasma or urine nitrate, cGMP, adhesion molecules, or IL-6 in patients.

Influence of treatment and smoking. The results in the corticosteroid- and ACE inhibitor–treated groups were not significantly different from the results in the untreated group, and the results in smokers were not significantly different from those in nonsmokers. Median plasma nitrate levels were 23.7 μM in the corticosteroid-treated group and 32.7 μM in the untreated group.

Exploratory data analysis. Factor analysis including data on levels of plasma and urine nitrate, cGMP, sVCAM-1, sICAM-1, sE-selectin, IL-6, and creatinine clearance yielded 4 factors with eigenvalues >1, explaining 84% of the total variation between these variables (Table 4). These included one factor showing large loadings on sE-selectin, sICAM-1, and to some extent plasma nitrate and sVCAM-1, a second factor showing large loadings on plasma and urine nitrate, a third on sVCAM-1 and IL-6, and a fourth on creatinine clearance and cGMP.

DISCUSSION

In order to further elucidate the pathophysiology of vascular dysfunction in SSc, we have studied markers

Table 3. Spearman's rank correlation coefficients for correlations between levels of p-NO, u-NO, urine cGMP, soluble adhesion molecules (sICAM-1, sVCAM-1, and sE-selectin), and IL-6 in 27 patients with systemic sclerosis and 27 matched controls*

Variable	u-NO	cGMP	sICAM-1	sVCAM-1	sE-selectin	IL-6
p-NO	0.483†	0.111	0.495‡	0.357§	0.409†	0.343†
u-NO		0.196	0.119	0.171	0.307§	0.280§
cGMP			0.219	0.088	0.181	0.233
sICAM-1				0.450†	0.593‡	0.360
sVCAM-1					0.446†	0.571‡
sE-selectin						0.324†

* See Table 2 for definitions.
† $P < 0.01$.
‡ $P < 0.001$.
§ $P < 0.05$.

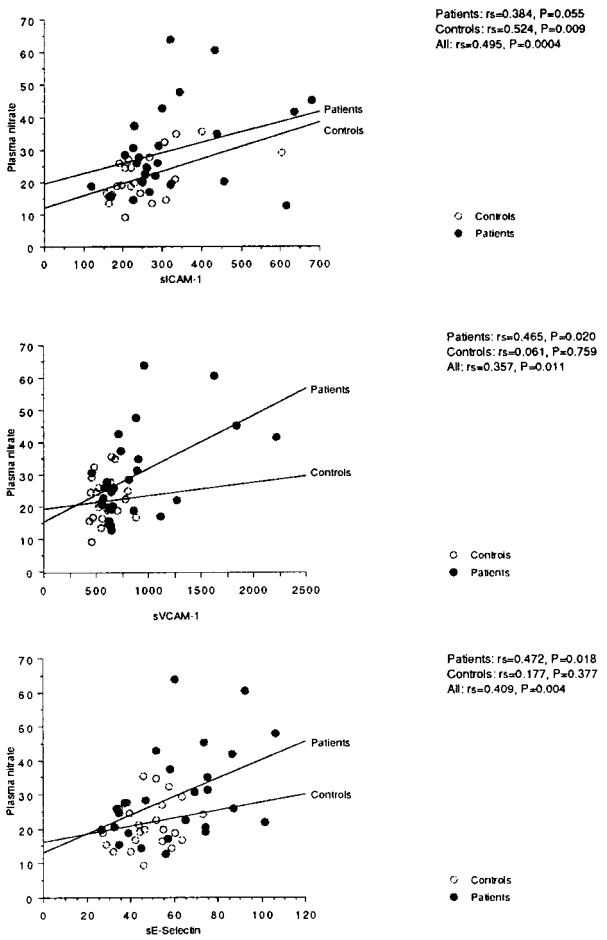

Figure 4. Scattergrams showing the correlation (Spearman's rank correlation coefficient) between levels of plasma nitrate and **A,** soluble intercellular adhesion molecule 1 (sICAM-1), **B,** soluble vascular cell adhesion molecule 1 (sVCAM-1), and **C,** soluble E-selectin in systemic sclerosis patients and age- and sex-matched controls (n = 27 in each group).

for the production of NO in the context of markers of endothelial activation. We found elevated levels of plasma nitrate as well as of sICAM-1, sVCAM-1, and sE-selectin. Moreover, the levels of sVCAM-1 and sE-selectin correlated strongly with levels of plasma nitrate in SSc patients. Exploratory factor analysis, when all variables were included, showed clustering of variables, consistent with the conclusion that activated endothelial cells are a likely source of elevated plasma nitrate. However, direct evidence for this is still lacking.

The increased levels of plasma nitrate observed in the present study indicate increased production of NO. This finding is corroborated by the increased levels of cGMP found in the urine, indicating that NO is active on the heme prosthetic group of guanylate cyclase. Kahaleh et al (17) observed low levels of NO in SSc patients, while Yamamoto et al (18) found increased NO levels as demonstrated in the present study. The discrepancy in these results could be explained by differences in the degree of inflammatory response, disease stage, and/or treatment of the patients, as well as lack of well-matched controls. The down-regulation of eNOS found by Kahaleh and Fan (19) does not necessarily result in any significant overall decrease in plasma NO since concomitant stimulation of iNOS by inflammatory cytokines might outweigh the effects of eNOS down-regulation (27).

Soluble E-selectin, sVCAM-1, and sICAM-1 are all produced and shed by endothelial cells upon stimulation by proinflammatory cytokines (28). E-selectin is expressed on and released from activated endothelial cells; sVCAM-1 is primarily released from endothelial cells, but can be produced by epithelium, dendritic cells, and macrophages. Soluble ICAM-1, on the other hand, can be produced by a variety of other cells including epithelium, leukocytes, hepatocytes, and smooth muscle cells (29). In SSc, VCAM-1 has been demonstrated on endothelial cells in skin biopsy specimens (30), while ICAM-1 beyond endothelial cells has been demonstrated on fibroblasts (30,31) and infiltrating mononuclear cells (32).

The significant correlation between increased levels of sE-selectin and sVCAM-1 and those of plasma nitrate in our patients implicates endothelial cells as an important common source of these factors. The lower correlation between plasma nitrate and sICAM-1 levels supports the conclusion that the sources of plasma nitrate and sICAM-1 are partially disparate. Factor analysis further implicates endothelial cells as a common

Table 4. Factor analysis with principal component analysis for p-NO, u-NO, urine cGMP, soluble adhesion molecules (sICAM-1, sVCAM-1, and sE-selectin), IL-6, and creatinine clearance in 27 patients with systemic sclerosis*

Variable	Factor			
	1	2	3	4
p-NO	0.441	0.803	–	–
u-NO	–	0.931	–	–
cGMP	–	–	–	0.933
sICAM-1	0.857	–	–	–
sVCAM-1	0.647	–	0.614	–
sE-selectin	0.902	–	–	–
IL-6	–	–	0.939	–
Creatinine clearance	–	–	–	−0.662

* See Table 2 for definitions.

source of these factors since they all load together as one factor. Importantly, E-selectin shedding is restricted to activated endothelial cells (33). Therefore, the increased levels of sE-selectin observed in this study are an indicator of activated endothelial cells. Expression of E-selectin on the vascular endothelium in skin biopsy specimens from our SSc patients is consistent with the finding of elevated sE-selectin and strongly supports the notion of an activated endothelium in SSc. The presence of leukocyte infiltration in close proximity to blood vessels indicates that the E-selectin is participating in directing the extravasation of leukocytes to the perivascular space. The results of immunoblotting studies of skin biopsy samples from the patients verified the presence of eNOS and iNOS. These data are consistent with the findings in studies of a related disease, systemic lupus erythematosus, in which NO production was also increased in combination with signs of activation of endothelial cells (i.e., increased expression of E-selectin and up-regulation of iNOS) (34,35).

Our data show a lack of positive correlation between levels of acute-phase reactants and IL-6 in the patients, suggesting that hepatocytes are not the main source of plasma IL-6. The loading of IL-6 and sVCAM-1 on a single factor in the factor analysis suggests that activated endothelial cells may further contribute to disease activity by producing IL-6. IL-1β and TNFα both induce iNOS and thus increase NO production. IL-1β and TNFα also induce production of adhesion molecules, IL-6, IL-1Ra, TGFβ, and IL-10. Although we demonstrated increased production of NO as well as adhesion molecules, IL-6, and IL-10, we could not detect any increase in circulating IL-1β and TNFα levels. Similar to our findings, Needleman et al were unable to detect IL-1β in sera from 78 SSc patients and controls, although they detected TNFα in 10 of 69 patient sera and 2 of 53 control sera (36). The failure to detect IL-1β might be due to local consumption, short half-life (37), or insensitive assays. Despite undetectable circulating levels, there is evidence of locally increased production of IL-1β and TNFα in involved tissues in SSc (38), which may be due to their production by infiltrating leukocytes such as those that we found in SSc skin. We detected IL-10 significantly more often in patients than in controls, which was consistent with the results of 2 previous studies (39,40). However, we found no significant correlation between levels of IL-10 and those of plasma nitrate, sE-selectin, sVCAM-1, sICAM-1, or IL-1Ra. Our data thus suggest that IL-10 plays a minor role in NO production and endothelial activation.

TGFβ1 was detectable in all patient and control sera, but levels were significantly lower in patients. This is consistent with the increased levels of nitrate since TGFβ1 inhibits iNOS. However, there was no significant correlation between levels of TGFβ1 and those of plasma nitrate or sE-selectin, suggesting a minor role for TGFβ1 in controlling the iNOS activity and adhesion molecule expression in SSc. Although TGFβ1 production and localization in affected tissues (41,42) as well as circulating levels (43,44) have been studied in SSc, there are no published reports on TGFβ1 production in relation to NO or sE-selectin in this disease.

In summary, in SSc patients with Raynaud's phenomenon, we found evidence of increased NO production, probably with a prominent contribution from endothelial cells activated by cytokines. Whether this increased production of NO should be regarded as a counterbalance to the increased endothelin levels and vascular tone demonstrated in earlier studies of SSc patients (12,13) or as a direct influence of inflammatory cytokines remains an open question.

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