Soluble CD95 concentrations are increased in patients with severe systemic lupus erythematosus, but not in their first degree relatives

M W van der Linden, T van Lopik, L A Aarden, R G J Westendorp, T W J Huizinga

Abstract

Objective—Plasma concentrations of soluble CD95 (sCD95) are raised in patients with systemic lupus erythematosus (SLE) before clinical relapses become manifest. Increased sCD95 concentrations may therefore be a familial characteristic that is associated with susceptibility to severe disease. To test this, sCD95 concentrations were measured in healthy first degree relatives of patients with severe and non-severe SLE.

Methods—Seventy seven first degree relatives of 26 patients with severe, and 72 relatives of 25 patients with non-severe lupus were studied. Controls were 42 first degree relatives of 17 patients with chronic cutaneous lupus erythematosus (CCLE) and 63 partners of the patients with their first degree relatives. Severe lupus was defined as both multi-organ disease and cyclophosphamide treatment, non-severe lupus as neither. Organ damage was assessed with the SLICC-ACR index, disease activity with SLEDAI.

Results—Soluble CD95 concentrations in relatives of patients with severe SLE were similar to those in relatives of patients with non-severe SLE, relatives of patients with CCLE, and controls (median (interquartile range) sCD95 concentration 0.59 (0.52–0.66) v 0.57 (0.50–0.63), 0.56 (0.51–0.71), and 0.55 (0.49–0.61) ng/ml, $p=0.25$, $p=0.94$, and $p=0.17$, respectively). Increased concentrations of sCD95, however, were found in patients with severe SLE compared with those in patients with non-severe SLE, patients with CCLE, and control relatives (0.77 (0.70–0.97) v 0.60 (0.54–0.67), 0.57 (0.54–0.71), and 0.57 (0.52–0.63) ng/ml, respectively, $p<0.001$). Concentrations of sCD95 were significantly correlated with damage index scores ($r_s=0.47$, $p<0.01$). Basic and clinical characteristics of patients with SLE, including SLEDAI scores, could not explain these observations.

Conclusion—Soluble CD95 concentrations are associated with severity of the disease and not with susceptibility for severe SLE.

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Systemic lupus erythematosus (SLE) is an autoimmune disease in which genetic factors have an important role. Antinuclear antibodies directed against antigenic components of the

cell nucleus, such as nucleosomal antigens, are a key feature of SLE. Autoantibodies are more often present in first degree relatives of patients with SLE than in the general population.^{1–4} This suggests that a strong non-specific humoral autoimmune response is a familial characteristic of SLE. Familial factors are also important in the severity of the disease.⁵

Antigens expressed in apoptotic cells are common targets for autoantibodies, which suggests that abnormalities of apoptosis are an important mechanism underlying autoantibody production and SLE.⁶ Gene transfer studies indicate that a molecule largely responsible for autoantibody production is CD95 (Apo-1, Fas), which is a key molecule in apoptosis regulation.⁷ Moreover, in the MRL-lpr/lpr mouse model for SLE, autoantibody production is associated with a genetic defect in CD95.⁸

Soluble CD95 (sCD95) molecules are formed as an alternative mRNA splice variant and as a proteolytically cleaved fragment of the transmembrane molecule CD95.^{9–10} Increased plasma concentrations of sCD95 have been reported in patients with SLE, and baseline plasma concentrations of sCD95 are higher in patients who relapse within six months than in patients who remain clinically quiescent.¹¹ Circulating sCD95 levels remain high in these patients at least until six months after relapse (van Lopik, personal communication). This suggests that patients who are prone to clinical relapses have high circulating levels of sCD95 as an inherent patient characteristic which may be genetically determined.

To test whether increased plasma concentrations of sCD95 are a familial characteristic of patients with SLE, we measured sCD95 in first degree relatives of these patients. Partners with their first degree relatives served as controls. Patients with chronic cutaneous lupus erythematosus (CCLE), with their first degree relatives, were studied as a second control group.

Methods

STUDY GROUP

First degree relatives of patients with severe and non-severe SLE were recruited. After approval by the institutional medical ethics board, patients fulfilling at least four of the American College of Rheumatologists' criteria for SLE¹² were identified from the Leiden University Medical Centre Rheumatology Clinic and outpatient clinic. Patients were classified as having severe SLE when treatment indication

Department of Clinical Epidemiology, Leiden University Medical Centre, The Netherlands
M W van der Linden
R G J Westendorp

Department of Rheumatology, Leiden University Medical Centre
T W J Huizinga

Department of Autoimmune Diseases, Central Laboratory of the Blood Transfusion Service, and Laboratory for Experimental and Clinical Immunology, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands
T van Lopik
L A Aarden

Correspondence to:
Dr T W J Huizinga,
Department of
Rheumatology, Building 1,
C4-R Leiden University
Medical Centre, PO Box
9600, 2300 RC Leiden, The
Netherlands
TWJHuizinga@
rheumatology.azl.nl

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Table 1 Demographic and clinical characteristics of families of patients with systemic lupus erythematosus (SLE), chronic cutaneous lupus erythematosus (CCLE), and controls

	Severe SLE*	Non-severe SLE*	CCLE	Controls
Number of families	26	25	17	21
Patients				
Male/female (n)	3/23	1/24	8/9	—
Age (years)	38 (3.4)	41 (2.5)	48 (2.7)	—
White/non-white (n)	18/8	24/1	17/0	—
Time since diagnosis (years)	8.0 (1.0)	13 (1.1)	14 (0.9)	—
Damage score†	4.9 (0.5)	1.2 (0.2)	—	—
Activity score‡	5.1 (1.1)	2.4 (0.5)	—	—
First degree relatives				
Number	77	72	42	63
Family size	3.1 (0.3)	3.2 (0.5)	2.5 (0.3)	2.4 (0.2)
Male/female (n)	33/44	33/39	15/27	30/33
Age (years)	41 (1.9)	47 (2.0)	38 (2.7)	49 (2.1)
White/non-white (n)	55/22	70/2	42/0	63/0

Results are shown as means (SEM).

*Severe SLE was defined by treatment with cyclophosphamide and renal/cerebral organ disease; non-severe SLE by 10 years' disease without cyclophosphamide treatment and involvement of at most one of these organ systems (see "Methods").

†Accumulated organ damage was measured by the SLICC/ACR Damage Index.

‡Disease activity at time of blood sampling was measured by SLEDAI.

for cyclophosphamide existed at any time since diagnosis for renal or cerebral disease, or both. Of 26 patients with an indication for cyclophosphamide treatment, three were eventually treated with azathioprine because of fertility concerns. Patients were classified as having non-severe SLE when they had never been treated with cyclophosphamide and had been free of multi-organ disease for at least 10 years. Patients who could be classified according to the criteria mentioned above were interviewed for eligibility and their first degree relatives (parents, brothers and sisters, and/or children) were also invited. One hundred and forty nine first degree relatives free of disease belonging to 26 patients with severe SLE and 25 patients with non-severe SLE participated. Accumulated organ damage was measured with the SLICC/ACR Damage Index, and disease activity with SLE the Disease Activity Index (SLEDAI).^{13 14}

If the patients agreed, their partners and the first degree relatives of the partners were invited to participate as controls. Twenty one partners and 42 of their first degree relatives were enrolled in a similar procedure as described above. As a second control group families of patients with histopathologically confirmed CCLE who had negative antinuclear factor serology on initial diagnosis were identified. All patients with CCLE had a disease course of at least 10 years to ensure that generalised disease had not developed. Forty two healthy first degree relatives belonging to 17 patients with CCLE were enrolled.

SOLUBLE CD95

The combination of CLB-CD95/2 and CLB-CD95/6 monoclonal antibodies directed against the extracellular portion of Fas was used. Microtitre plates (Nunc, Denmark) were coated with 100 µl/well CLB-CD95/2 in 0.1 M NaHCO₃/Na₂CO₃ buffer (pH 9.6) overnight at room temperature. Coated plates were washed five times with phosphate buffered saline (PBS)/Tween-20 (PBST). Samples and standards were diluted in a high performance enzyme linked immunosorbent assay (ELISA) buffer (CLB, Amsterdam, The Netherlands) and

100 µl of each sample dilution was added to the plate. To each sample dilution, 10 µl of a 10 µg/ml solution of biotin coupled CLB-CD95/6 was added and the plate was incubated for two hours at room temperature. After five washes with PBST 100 µl of streptavidine-poly horseradish peroxidase (CLB, Amsterdam, The Netherlands) diluted 1:10 000 in PBS containing 2% whole milk was incubated for 30 minutes at room temperature. The plates were washed five times with PBST and developed with 100 µl substrate solution (0.1 mg/ml 3,5,3',5'-tetramethylbenzidine, Merck, Darmstadt, Germany) containing 0.003% H₂O₂ in 0.11 M NaAc (pH 5.5) for 10 minutes. The enzyme reaction was stopped with 100 µl 2 M H₂SO₄. Plates were read at 450 nm in a titertek Multiskan reader (Labsystems Multiskan Multisoft, Helsinki, Finland).¹⁵

STATISTICAL ANALYSIS

Comparisons between multiple groups were first tested with the Kruskal-Wallis test to detect differences between groups. If a difference was present, Mann-Whitney's test was then used to identify groups responsible for the difference. Spearman's coefficient was used to test correlation between variables. These non-parametric tests do not make assumptions about the distribution of the data. In some analyses, adjustment for confounding variables was performed using linear regression. In these analyses the confounding variable (age, serum creatinine concentration) was introduced as a covariate in the regression equation that models concentration of sCD95 according to group—that is, patients, relatives of patients, or controls.

Results

DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF PATIENTS, FIRST DEGREE RELATIVES, AND CONTROLS

Table 1 shows the demographic and clinical characteristics of those studied. Families of patients with CCLE and control families were all white, but nine families of patients with SLE were not. Patients with severe SLE, their first degree relatives, and first degree relatives of patients with CCLE were younger than the controls. More of them were female than among the controls. As a result of patient classification, organ damage and disease activity were higher in the patients with severe SLE than in those with non-severe SLE. Among the 20 patients with severe SLE who were not currently treated with cyclophosphamide at the time of blood sampling or less than six months before, 14 (70%) had active disease necessitating treatment with steroids either alone or in combination with azathioprine. Organ damage in these 20 patients was similar to that in the six patients with severe SLE who were currently being treated with cyclophosphamide (data not shown).

Among relatives of patients with SLE, there was no difference in sCD95 concentration between white and non-white subjects (median (25th centile-75th centile) sCD95 concentration 0.58 (0.51-0.64) *v* 0.58 (0.50-0.66)

Table 2 Plasma concentration of sCD95 in first degree relatives of patients with systemic lupus erythematosus (SLE), first degree relatives of patients with chronic cutaneous lupus erythematosus (CCLE), and controls

	Relatives of patients with SLE	Relatives of patients with CCLE	Relatives of partners
Number	149	42	42
Soluble CD95 (ng/ml)			
Median	0.58	0.56	0.57
(Interquartile range)	(0.51–0.65)	(0.51–0.71)	(0.52–0.63)

No statistically significant differences in sCD95 concentration between these groups were found ($p > 0.4$, Mann-Whitney tests).

Table 3 Plasma concentration of sCD95 in patients with systemic lupus erythematosus (SLE), patients with chronic cutaneous lupus erythematosus (CCLE), and controls

	Severe SLE†	Non-severe SLE†	CCLE	Partners of patients	Relatives of partners
Number	26	25	17	21	42
Soluble CD95 (ng/ml)					
Median	0.77**	0.60*	0.57	0.53	0.57
(Interquartile range)	(0.70–0.97)	(0.54–0.67)	(0.54–0.71)	(0.46–0.59)	(0.52–0.63)

* $p = 0.008$ compared with partners, $p = 0.324$ compared with relatives of partners.

** $p < 0.001$ compared with patients with non-severe SLE, patients with CCLE, partners, and relatives of partners.

†Severe SLE was defined by treatment with cyclophosphamide and renal/cerebral organ disease; non-severe SLE by 10 years' disease without cyclophosphamide treatment and involvement of at most one of these organ systems (see "Methods").

ng/ml, $p = 0.95$). Among the patients, there was also no such difference (data not shown). There was a moderate correlation between age and sCD95 concentrations ($r_s = 0.4$, $p = 0.002$, among control subjects). No association between sCD95 concentrations and sex was found (concentration of sCD95 in male controls 0.54 (0.50–0.63) *v* 0.57 (0.46–0.61) ng/ml in female controls, $p = 0.48$).

SOLUBLE CD95 IN FAMILIES OF PATIENTS WITH SLE, FAMILIES OF PATIENTS WITH CCLE, AND CONTROLS

To examine whether an increased sCD95 concentration is a familial susceptibility factor for SLE and CCLE, plasma sCD95 concentrations were measured in first degree relatives of the patients and controls. Table 2 indicates plasma concentrations of sCD95 in first degree relatives of patients with SLE, first degree relatives of patients with CCLE, and healthy controls. To avoid a possible influence of shared environment between patients and partner controls, only the first degree relatives of the partners are presented in table 2. There was no difference between the sCD95 concentrations in first degree relatives of patients with SLE and the first degree relatives of the partners (0.58 (0.51–0.65) *v* 0.57 (0.52–0.63) ng/ml, $p = 0.85$).

SOLUBLE CD95 IN RELATION TO SEVERITY OF SLE

It was first investigated whether increased plasma concentrations of sCD95 are a familial disease characteristic of severe SLE, as defined by treatment with cyclophosphamide and renal/cerebral organ disease. Soluble CD95 concentrations in first degree relatives of patients with severe SLE were similar to those in first degree relatives of patients with non-severe SLE (0.59 (0.52–0.66) *v* 0.57 (0.50–0.63) ng/ml, $p = 0.25$). Soluble CD95 concentrations were also similar in first degree relatives of patients with severe SLE and in first degree relatives of patients with CCLE (0.59

(0.52–0.66) *v* 0.56 (0.51–0.71) ng/ml, $p = 0.94$). When adjustment for the effect of age was made, similar results were obtained (linear regression, data not shown).

It was then investigated whether increased plasma concentrations of sCD95 are a non-familial disease characteristic of disease severity, as defined by treatment with cyclophosphamide and renal/cerebral organ disease. Therefore plasma concentrations of sCD95 were measured in patients with severe and non-severe SLE. Plasma concentrations of sCD95 were higher in patients with SLE than in their own first degree relatives (0.69 (0.58–0.71) *v* 0.58 (0.51–0.65) ng/ml, $p < 0.001$). Table 3 compares plasma concentrations of sCD95 in patients with severe and non-severe SLE with those in patients with CCLE and controls. Increased plasma concentrations of sCD95 in patients with severe SLE compared with those in patients with non-severe SLE, patients with CCLE, and controls were seen (Kruskal-Wallis and Mann-Whitney tests, $p < 0.001$). This association between sCD95 concentration and disease severity in patients was unaffected after adjustment for the effect of age ($p < 0.001$). A trend towards increased sCD95 concentrations in patients with non-severe SLE when compared with controls was seen ($p = 0.07$). After adjustment for the effect of age this trend was stronger ($p = 0.02$). No significant difference in sCD95 concentration was found between patients with CCLE and controls ($p = 0.18$).

Disease activity may have an influence on increased sCD95 concentrations in patients with severe and non-severe SLE. Indeed, sCD95 and SLEDAI values were significantly correlated (Spearman's correlation coefficient, $r_s = 0.40$, $p < 0.01$). Therefore, the comparison of sCD95 concentrations in patients with severe SLE with those in patients with non-severe SLE, as presented in table 3, was adjusted for SLEDAI scores. The results were similar (data not shown). In line with these analyses, a positive correlation between sCD95 concentration and organ damage, as measured by SLICC/ACR Damage Index values, was found. This remained essentially unchanged after adjustment for SLEDAI scores (regression, $r_s = 0.47$, $p < 0.001$, and $r_s = 0.30$, $p = 0.016$, respectively).

Specific disease manifestations associated with high SLICC/ACR Damage Index values were SLE nephritis and neuropsychiatric SLE. Higher sCD95 concentrations were found in patients with nephritis than in those without (0.72 (0.68–0.94) *v* 0.62 (0.56–0.74) ng/ml, $p = 0.003$). Also, higher sCD95 concentrations were found in patients with neuropsychiatric SLE than in those without (0.77 (0.69–0.98) *v* 0.66 (0.58–0.75) ng/ml, $p = 0.014$). However, these differences disappeared when patients with severe SLE and patients with non-severe SLE were analysed separately (patients with severe SLE: nephritis *v* non-nephritis, $p = 0.324$, neuropsychiatric SLE *v* non-neuropsychiatric SLE, $p = 0.71$; patients with non-severe SLE: nephritis *v* non-nephritis, $p = 0.58$, neuropsychiatric SLE *v* non-neuropsychiatric SLE, $p = 0.89$).

Increased sCD95 concentrations in patients with severe SLE may be explained by renal function impairment. However, analysis of sCD95 concentrations adjusted for serum creatinine concentrations yielded results similar to the unadjusted analysis ($p < 0.001$). Also, the combined effects of age and creatinine concentrations did not explain the increase in sCD95 concentrations in patients with severe SLE compared with patients with non-severe SLE ($p = 0.001$).

To control for a possible influence of current treatment of patients with cytotoxic drugs (cyclophosphamide, prednisone, and/or azathioprine) the analysis was restricted to patients not currently (< 6 months) using these drugs. The plasma concentration of sCD95 in patients with severe SLE without current cytotoxic treatment ($n = 20$) was higher than in patients with non-severe SLE without current cytotoxic treatment (such as azathioprine or prednisone) ($n = 17$) (0.76 (0.65 – 0.92) *v* 0.60 (0.53 – 0.67) ng/ml, $p = 0.012$).

Discussion

In this study no evidence for an increased concentration of sCD95 in first degree relatives of patients with SLE was found. Nevertheless, increased concentrations of sCD95 in patients with severe SLE were found. This suggests that an increase in sCD95 concentrations is associated with severity of the disease, and not with familial susceptibility for SLE.

Soluble CD95 concentrations were highest in patients with severe SLE, as defined by treatment with cyclophosphamide and renal/cerebral organ disease, and a correlation between sCD95 and the SLICC/ACR Damage Index was found. Therefore, an increased concentration of sCD95 is interpreted as a characteristic of severe SLE. This is in agreement with a transverse study that showed a correlation between sCD95 concentrations and the SLICC/ACR Damage Index values,¹⁶ and also with our transverse and longitudinal studies of a different set of patients with SLE that showed an increase in sCD95 concentrations even before clinical exacerbations become manifest.^{11–15} Nevertheless, we found an association between sCD95 concentrations and current disease activity as measured by the SLEDAI. This association is in line with earlier reports,^{15–17} though other groups found normal sCD95 concentrations in patients with SLE, probably owing to differences in assay characteristics.^{18–21} It is noteworthy that disease activity in itself cannot explain the association of sCD95 with severity and organ damage in SLE.

To avoid misclassification of patients with severe SLE into the non-severe SLE group the criterion of 10 years' disease without involvement of more than one of the renal and cerebral organ systems, and without cyclophosphamide treatment was used. In a 10 year follow up study Ginzler *et al* showed that of 29% of patients with SLE who eventually develop lethal complications, the vast majority do so within the first five years after diagnosis.²²

Increased concentrations of sCD95 may be explained by cytotoxic drugs, which are known from experimental settings to induce apoptosis.²³ This may subsequently lead to the release of sCD95 from apoptotic cell fragments. However, an analysis restricted to those without cytotoxic drugs excludes the substantial influence of drugs.

Several mechanisms of action of sCD95 in the pathogenesis of severe SLE may be at play. Soluble CD95 may block the action of the CD95 ligand (CD95L), thereby preventing CD95-CD95L interaction and apoptosis initiation.⁷ Increased concentrations of sCD95 might thus give rise to autoimmunity by inappropriate survival of autoreactive cell populations—for example, immature B lymphocyte cell lines in peripheral lymph nodes. This mechanism is in line with the increased autoantibody production observed in the MRL-lpr/lpr mouse model and in its human counterpart, the genetic CD95 defect leading to the Canale-Smith syndrome.²⁴ However, the genetic background of SLE is not identical with the monogenetic Canale-Smith syndrome. Other defects, such as apoptosis defects in B cells, inappropriately low clearance of apoptotic bodies, and increased production of B cell stimulating cytokines, such as interleukin 8 and interleukin 10, may contribute to increased production of autoantibodies in SLE. Increased sCD95 concentrations in SLE may also result from impaired clearance of apoptotic cell fragments. In this hypothesis, activation of lymphocytes leads to up regulation of CD95. Subsequently, the resulting activation-induced cell death of CD95 positive cells overloads the body's clearance capacity.^{25–26}

In conclusion, plasma concentrations of sCD95 are not increased in first degree relatives of patients with SLE. There is, however, an increase of sCD95 in patients with severe SLE, independent of concomitant factors such as disease flares and current immunosuppressive drugs.

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