Patients with Systemic Lupus Erythematosus with High Plasma Levels of sFas Risk Relapse

Thea van Lopik¹
Marc Bijl²
Margreet Hart¹
Leonie Boeije¹
Tom Gesner³
Abla A. Creasy³
Cees G. M. Kallenberg²
Lucien A. Aarden¹
Ruud J. T. Smeenk¹

¹ CLB, Sanguin Blood Supply Foundation, Laboratory of Experimental and Clinical Immunology, Academical Medical Center, University of Amsterdam, Amsterdam, The Netherlands
² Department of Clinical Immunology, University Hospital, Groningen, The Netherlands
³ Chiron, Emeryville, CA, USA.

J Rheumatol 1999;26:60-7
Chapter II

Summary

We related soluble Fas (sFas) levels to the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) in longitudinal series of plasma samples of SLE patients to evaluate the relation between excessive production of sFas and disease activity. Therefore, 21 monoclonal antibodies against Fas were raised. Two of these were used to develop and validate a sensitive sandwich ELISA for the longitudinal analysis of sFas levels in plasma of 30 patients and 25 controls. At the start of follow-up, a significant elevation (p<0.0001) was found in sFas levels in SLE (1167±347 pg/ml sFas) compared to controls (618±98 pg/ml sFas). Also, at the start of the follow-up a significant difference (p=0.0028) existed between patients who were going to have a relapse (1236±402 pg/ml sFas) during follow-up and patients who were not (809±276 pg/ml sFas). While sFas did not fluctuate with disease activity in individual patients, we found a strong correlation (r=0.75, p<0.0001) between sFas and SLEDAI, but only at the time of relapse, when we analysed the patients as a group. In individual SLE patients, sFas does not fluctuate with disease activity. However, patients with high plasma levels of sFas are at risk of relapse.

Introduction

A role for Fas in the pathogenesis of systemic lupus erythematosus (SLE) is cogently demonstrated in murine models of autoimmunity, the MRL-lpr/lpr -mouse and the MRL-gld/gld-mouse. The phenotypic characteristics of these single gene mutations in the Fas gene and the Fas ligand gene, respectively, together with an appropriate genetic background, mimic many of the features of human SLE [1-3].

The Fas protein denotes the CD95 receptor and is a 45-48 kDa type 1 membrane protein with sequence homology to the TNF-receptor family [3-5]. Membrane Fas (mFas) is expressed on many different tumour cells [6,7] and on various normal human tissues [8,9]. Triggering of mFas by its ligand results in rapid induction of programmed cell death, apoptosis, in susceptible cells [8,10]. The tissue distribution of Fas and Fas ligand suggests that the Fas receptor / ligand system plays an important role in various aspects of mammalian development, especially in the homeostasis of the immune system [11-18]. The susceptibility of cells to apoptosis-induced cell death is regulated through their state of activation, activity of down-stream ICE-like proteases (caspases) and activity of members of the bcl-2/ bax family [19-25].

Following in vitro activation of T-cells isolated from peripheral blood mononuclear cells (PBMC) of SLE patients, no defects in up regulation of the receptor or in the ability of the activated cells to undergo apoptosis are detected, suggesting that in the majority of SLE patients it is unlikely that SLE is caused by defects in the Fas receptor [26]. Two studies reveal human Fas messenger RNA variants capable of encoding sFas molecules lacking either the transmembrane domain or the extracytoplasmic region [4,27]. Patients with SLE display elevated levels of sFas [4,28-31], but not all studies can confirm this elevation.
Soluble isoforms of cell surface receptors, generated either by proteolytic cleavage of the transmembrane form or by alternative splicing, regulate receptor function in a number of biological systems. Such soluble receptors most commonly retain ligand-binding activity and compete for ligand [34-36]. A soluble form of the Fas receptor might prevent binding of FasL to Fas thereby blocking Fas-mediated apoptosis. When Fas mediated apoptosis is disturbed, autoreactive lymphocytes might persist and give rise to excessive autoantibody levels in the blood. Therefore, the rise in anti-DNA antibodies just before a disease exacerbation in SLE [37] might thereby be preceded by a rise in soluble Fas levels. In this study we tested this hypothesis by studying sFas levels in relation to SLEDAI scores, in longitudinal plasma samples.

Materials and methods

Generation of monoclonal antibodies against the Fas receptor.

Human recombinant Fas (rFas) cloning and expression were as follows: the cDNA for the extracellular domain of Fas was obtained by RT-PCR. Primers were designed based on the published sequence [5]. The 3' primer was designed to encode for a C-terminal glu-glu epitope tag [38]. The cDNA was ligated into the pAcC13 vector [39]. Two microgram plasmid DNA and 0.5 µg linearized wild type viral DNA were cotransfected into Sf9 cells [40]. Recombinant virus was isolated by plaque purification [41]. Supernatants from Sf9 cells were 10 times concentrated and passed over an anti-glu-glu/protein G column. Recombinant Fas was eluted with glu-glu peptide and peak fractions were pooled  and dialyzed versus phosphate buffered saline (PBS). Purity was more than 95%. Protein determination was made by amino acid analysis.

Two balb-c mice were immunised and 2 times boostered with 10 µg of this preparation of rFas in montanide (Seppic, France). Mouse sera were tested for the presence of Fas antibody by radio immunoassay (RIA). In that assay mouse sera were incubated with rat-anti-mouse kappa-light chain coupled to Sepharose (Pharmacia, Sweden) in the presence of 125I labeled rFas. Spleen cells of the mouse displaying a clear anti-Fas signal were fused with non-secreting mouse myeloma cells Sp2/0 under standard conditions [42]. Positive wells were cloned twice by limiting dilution cloning and 21 stable Fas antibody producing clones were isolated. Culture supernatants of these clones were concentrated and affinity purified on protein-A Sepharose CL-4B (Pharmacia, Uppsala, Sweden). Reactivity of these antibodies with wild type mFas was analysed by FACS analysis. Anti-Fas antibodies were biotinylated following manufacturer's instruction using LC-biotin-N-hydroxysuccinimide ester (Pierce chemical Co., Rockford, IL) and assayed on Jurkat and SKW6.4 cells at 5 µg/ml and 200.000 cells per sample. Streptavidin-coupled fluorescein isothiocyanate (Becton Dickinson Immunocytochemistry Systems, San José, CA) was used (1:50) as fluorescent marker. All 21 anti-Fas antibodies reacted with Jurkat and SKW6.4 cells. At least six of them showed bioactivity: CLB-CD95/10, /15 and /18
induced apoptosis while CLB-CD95/2, CLB-CD95/6 and CLB95/19 prevented apoptosis induced by either anti-Fas antibody or TCR triggering of Jurkat cells. The monoclonal antibodies to Fas will be described in detail elsewhere.

**ELISA for soluble Fas.**

To develop a sensitive sandwich ELISA for the detection of soluble Fas different combinations of monoclonal anti-Fas antibodies were evaluated. The use of CLB-CD95/2 to coat plates and biotin-coupled CLB-CD95/6 as a conjugate resulted in the most sensitive assay. The ELISA was performed as follows. Microtiter plates (Nunc-Immuno plate Maxisorp surface, Nunc, Denmark) were coated with 100 µl/well CLB-CD95/2 (2 µg/ml) in 0.1 M NaHCO3/Na2CO3 buffer (pH=9.6) overnight at room temperature. Coated plates were washed 5 times with 100 µl/well of phosphate buffered saline (PBS) containing 0.02% Tween-20 (PBST). Samples and standards were diluted in high performance ELISA (HPE) 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 g/ml solution of biotin-coupled CLB-CD95/6 was added and the plate was incubated for 2 hours at room temperature. After 5 washes with 100 µl/well PBST 100 µl of streptavidine-polyHRP (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 5 times with 100 µl/well 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).

**Patients and plasma samples.**

This study concerned a cohort of SLE patients fulfilling the 1982 revised ACR criteria [43] who participated in prospective longterm clinical follow-up studies [44,45]. Patients were evaluated at least every three months at the outclinic. The SLE disease activity index [46] was calculated at every outclinic visit from signs and symptoms and routine laboratory tests. Special attention was paid to the occurrence of infections in order to discriminate between infection and disease activity. Dosages of drugs were recorded and exacerbations were defined as described previously [43,44]. Blood samples were drawn in EDTA monthly and plasma was stored at -80°C. Exacerbations are defined as described previously [37] and are shown in table 1. For this study the first 20 patients (median age: 30.5±9 years, 19 females, 1 male) who experienced an exacerbation (12 major and 8 minor) during this prospective observation were randomly selected. Analysed were blood samples from 6 monthly time points before the exacerbation (t=-6,-5,-4,-3,-2,-1), from the moment of the exacerbation (t=0), and from one month afterwards (t=1). In addition, from the patients who did not experience an exacerbation during the follow-up studies, the first 10 patients
soluble Fas in SLE

(median age: 42±11 years, 9 females, 1 male) were randomly selected. During that period doses of immunosuppression and/or corticosteroids were not changed. From those patients

Table 1:

Criteria for major and minor disease exacerbations in SLE:

Criteria for major exacerbation: fulfilling **one or more** of the following *:

1. **severe renal disease:**
   a. recent renal biopsy showing active proliferative lupus nephritis (>50% of glomeruli affected, and/or
   b. decrease of creatinin clearance of >25% within 4 months, accompanied by an active sediment (>5 ery's, h.p.f., and/or casts) and by proteinuria of >0.5 gram/day)
2. **severe central nervous system disease:** seizures, cerebral vascular accident, coma, transverse myelitis, psychosis, choreathetosis, central nerve palsy
3. **hematological disease:** hemolytic anemia (Hb<3.8 mmol/l) and/or thrombocytopenia (<50x10⁹/l)
4. **severe serositis:** pericarditis with (impending) tamponade and/or massive pleural effusion
5. **uveitis and/or retinal vasculitis**
6. **myocarditis with arrhythmia and/or congestive heart failure**
7. **severe myositis with proximal muscle weakness**
8. **lung involvement with hemoptysis**
9. **major vasculitis:** with ulcerations and/or mononeuritis multiplex
10. **miscellaneous:** fever (>38°C rectally), serositis, hemolytic anemia (>3.8 mmol/l) or thrombocytopenia (>50x10⁹/l), all without improvement after prednisolone in a maximum dosage of 30 mg/day during at least one week

Criteria for minor exacerbation: fulfilling **all** of the following items:

1. increase of activity index by ≥ 2 points within 6 months with a minimal activity score of 3 points accompanied by:
2. the clinically judged necessity to start prednisolone at a dosage of at least 10 mg/day, or to increase the prednisolone dosage with ≥ 5 mg/day, or to start with anti-malarials, or immunosuppressive drugs, and:
3. not fulfilling the criteria for a major exacerbation.

- Only features occurring within 2 weeks of the outclinic visit or admission under consideration are taken into account

6 consecutive monthly samples were analysed for sFas. As a control group, samples from 25 healthy laboratory donors (median age: 42±12 years, 14 females, 11 males) were assayed for sFas.
Statistical analysis.
Comparisons between patient groups and healthy donors were calculated using the Mann-Whitney U test (2-tailed). Spearman's test was applied for detecting correlation between SLEDAI and sFas. P values less than 0.05 were considered significant.

Results

Combination of CLB-CD95/2 as coating antibody with biotinylated CLB-CD95/6 as conjugate resulted in a sensitive sandwich ELISA with a detection limit for recombinant Fas of 4 pg/ml (twice the background). Titration of sera of 5 healthy blood bank donors in this assay resulted in dose-response curves parallel to the rFas curve as demonstrated in figure 1, indicating comparable affinity for rFas and sFas. The median sFas level in 62 healthy donors was 647±176 pg/ml. The mean interassay variation of this ELISA was 15.9 % and the mean intra assay variation was 6.7 %. Assay readings were not affected by the use of plasma instead of serum (data not shown).

![Image](image.png)

**Figure 1**: Comparison of recombinant Fas and soluble Fas in serum samples of 5 healthy blood donors (bd5). Soluble Fas levels were determined in duplicate. Detection limit was 4 pg/ml.

Soluble Fas levels at t=-6 were analysed for 25 control donors (C) and the 30 SLE patients (S) (figure 2a). The latter were divided in patients who developed a clinical exacerbation (E) six months later, and those with quiescent disease (Q) (figure 2a). Soluble Fas was higher in all SLE patients relative to controls (1167±347 versus 618±98 pg/ml, p<0.0001). Most important, patients who are going to have a relapse, have higher sFas levels than those who are not (1236±402 versus 809±276 pg/ml, p=0.028), while
SLEDAI scores (figure 2b) and immunosuppressive medication did not differ between both groups at that time point. At all other time points (t=-5, -4, -3 , -2, -1, 0, 1) the result of this analysis was identical (data not shown).

![Figure 2](image.png)

**Figure 2**: (A) Comparison of recombinant sFas levels at the start of the follow-up between controls (C), 30 SLE patients (S), the subgroup of SLE patients who experienced an exacerbation during follow-up (E), and the subgroup of exacerbation patients who were exacerbation-free (Q) during follow-up. ○: quiescent patients, ●: patients who were going to have a relapse at t = 0. P values of comparisons between the different groups are given. (B) Comparison of SLEDAI scores of the subgroup of exacerbation patients (E) and quiescent patients (Q) at start and at the time of a relapse (for exacerbation patients) or at the end of follow-up (for quiescent patients).

Individual sFas curves for each patient group were analysed and no correlation was found between sFas and SLEDAI. In figure 3a, three examples of sFas curves and SLEDAI scores of exacerbation patients (E1,E2,E3) are shown. Most sFas curves were similar to the one of patient E1, but different curves existed too, like E2 and E3. Furthermore, in figure 3b, 3 examples of non-exacerbation patients (Q1,Q2,Q3) and in figure 3c, 3 examples of controls (C1,C2,C3) are shown. When we looked at the SLE patients as a group we found a strong correlation (r=0.75, p<0.0001, n=30) between SLEDAI and sFas at t=0, the moment of relapse (figure 4a), but no correlation at all at t=−6, when all patients were quiescent (figure 4b). The most interesting aspect of our study is that there was also a correlation between soluble Fas levels at t=−6 and SLEDAI scores at t=0 (figure 4c). These results suggest that sFas levels in SLE patients are rather constant, and might be related to the severity of a coming relapse.

**Discussion**

For the retrospective analysis of soluble Fas (sFas) levels in SLE blood samples from a prospectively designed longitudinal study, we developed a sensitive and reproducible sandwich ELISA using a combination of 2 monoclonal anti-Fas antibodies, CLB-CD95/2
and CLB-CD95/6. Affinities of the anti-Fas antibodies for recombinant Fas and blood sFas were comparable and levels in serum and plasma of the same donor were equal.

![Figure 3: Soluble Fas curves for (A) 3 exacerbation patients (E1-3); (B) for 3 non-exacerbation patients (1-3); and (C) (opposite) for 3 controls (C1-3). sFas values (●) are given on the left y-axis and SLEDAI values (□) on the right y-axis.](image)

We analysed sFas in plasma samples in patients with SLE. We found that soluble Fas levels were elevated in patients compared to healthy controls, as in 5 other studies [4,28-31]. A difference in patient population is not a very likely explanation why Knipping et al. [32] and Goel et al. [33] did not find elevated sFas levels in SLE, as both investigators
analysed both active and inactive patients. In addition, in patients with quiescent SLE, sFas levels were higher than in controls. Since the assay used by Knipping et al [32] has a detection limit of 2 ng/ml, which also appears to be the detection limit in the assay used.

(right) **Figure 4**: correlation for the group of 30 SLE patients between sFas levels and SLEDAI scores. ●: exacerbation patients, ○: quiescent patients. (A) correlation between sFas and SLEDAI at time $t = 0$ ($r=0.75, p<0.0001$). (B) correlation between sFas and SLEDAI at time $t = -6$ ($r = 0.31, p = \text{not significant}$). (C) correlation between sFas at $t = -6$ and SLEDAI $t = 0$ ($r = 0.58, p = 0.0009$).
by Goel et al [33] it is likely that these assays are not sensitive enough to detect elevated serum levels of sFas.

We observed that already at the start of the follow-up there was a significant difference in sFas levels between the group of patients who were going to have an exacerbation later on, and the group of patients who were not, although both groups were clinically quiescent at that stage and did not differ in their use of immunosuppressives and/or corticosteroids.

This difference was found for all time points analysed, including the moment that all 20 patients had active disease \((t=0)\). This confirms the study by Jodo et al. [28], who compare sFas levels between active SLE (SLEDAI >9) and inactive SLE (SLEDAI<10).

In individual patients we did not find a correlation between sFas and SLEDAI, but sFas did correlate to SLEDAI scores in the whole group of patients, when we looked at one time point \((t=0)\). This shows that the relation between disease activity and sFas is completely dependent on the patient population blood samples are drawn from, i.e., the greater the number of patients with active disease, the greater the likelihood there will be a relation between sFas and SLEDAI. That is why Jodo et al. [28] found a correlation between sFas and SLEDAI also.

Remarkably, sFas levels at \(t=-6\) correlate with the SLEDAI score at \(t=0\), indicating that sFas levels in SLE patients are rather constant, and might be related to the severity of a coming relapse. Renal abnormalities, as occur in SLE patients may influence levels of small sized circulating proteins in various ways. Proteinuria may result in loss of sFas from the circulation, whereas renal failure may decrease, to some extent, the clearance of this polypeptide. We found no relation between creatinin clearance or proteinuria and soluble Fas levels. In addition, we compared sFas values of patients who had an exacerbation with renal involvement \((n=7)\) to patients who had not \((n=13)\) and found no significant difference in sFas levels between patients with renal involvement and others (data not shown).

Although sFas levels in SLE patients were elevated, the amounts of sFas circulating are fairly low, rendering a pathophysiological role for soluble Fas doubtful: Papoff et al. [27] found that a concentration of 20-100 ng/ml sFas is necessary for inhibition of apoptosis in two cell lines. However, the expression levels of Fas ligand on cells certainly play an important role. Furthermore, it cannot be excluded that sFas is produced in specific tissues in larger amounts and exerts its effects there locally.

Elevated soluble Fas levels have been measured in tissues of in a considerable number of diseases [4], [28-31], [32-34,47-52], [53-58]. From this it can be concluded that elevation of soluble Fas is not specific for rheumatic diseases.

Regarding the source of sFas in serum, it is suggested by Cheng et al. [4] and by Papoff et al. [27] that sFas is produced by alternative splicing. However, this could not be confirmed by Rose et al. [31] who do not find a difference in the production of mRNA for soluble Fas between controls and SLE patients. Possibly, the Fas receptor is released from cells upon immune activation, as is described for the TNF receptors and the endothelial
adhesion molecules sICAM1, E-selectin and sVCAM1 after recombinant human tumor necrosis factor treatment of cancer patients [59]. We conclude that sFas levels are elevated in SLE patients, but in individual patients sFas does not fluctuate with disease activity. Our results indicate that sFas levels are constantly elevated and that sFas levels might be indicative for the severity of an exacerbation. It remains to be determined from which cells sFas originates and whether there is a relation with activation markers on immune cells. The question at what moment sFas levels get elevated in active SLE is currently under investigation in a prospective study, covering more than 6 months prior to an exacerbation.

References

Soluble Fas in SLE


55. Munker, R., Midis, G., Owen-Schaub, L., and Andreff, M. 1996. Soluble Fas (CD95) is not elevated in the serum of patients with myeloid leukemias, myeloproliferative and myelodysplastic syndromes. Leukemia 10:1531.


