Anti-CD3-induced and anti-Fas-induced apoptosis in systemic lupus erythematosus (SLE)

Marc Bijl
Gerda Horst
Pieter C. Limburg
Cees G. M. Kallenberg

1 Department of Clinical Immunology, University Hospital, Groningen, The Netherlands
2 Department of Pathology and Laboratory Medicine, University Hospital, Groningen, The Netherlands

Summary

Disturbances in apoptosis or in the clearance of apoptotic material might result in increased presentation of autoantigens which could be relevant to the pathogenesis of SLE. Data concerning defects in apoptosis in SLE are conflicting. To determine whether intrinsic defects in apoptosis induction occur in SLE irrespective of disease activity we examined anti-CD3 and anti-Fas induced apoptosis in vitro in SLE patients with inactive disease. Isolated peripheral blood lymphocytes (PBL) from 13 SLE patients and 14 healthy controls were incubated with anti-CD3, and, subsequently, after up-regulation of membrane Fas following anti-CD3 incubation, with anti-Fas. Expression of Fas and levels of apoptosis as detected by annexin V and propidium iodide staining were assessed by flow cytometry before and after the respective incubations. Fas expression on freshly isolated lymphocytes of SLE patients was increased whereas levels of circulating apoptotic cells were comparable between patients and controls. Stimulation with anti-CD3 resulted in up-regulation of membrane Fas in patients and in controls. In vitro induction of apoptosis by anti-CD3 as well as by anti-Fas occurred both in SLE patients and controls, and was higher in SLE patients after incubation with both anti-CD3 as well as with anti-Fas. We conclude that Fas expression and in vitro induction of apoptosis is increased in SLE even in the absence of disease activity.

Introduction

SLE, the prototype of a systemic autoimmune disease, is associated with various immunologic abnormalities such as increased number of activated B lymphocytes and production of multiple autoantibodies. Autoantibody production has been shown to be selective, T cell dependent, and antigen-driven. Even in inactive disease peripheral blood lymphocytes (PBL) show signs of activation [1]. Persistent autoantigen presentation may be responsible for this continuous state of lymphocyte activation. Presentation of cryptic antigens and modification, including cleavage and phosphorylation, can occur during apoptosis resulting in autoantibody production [2,3]. Therefore, it can be speculated that increased apoptosis of peripheral blood mononuclear cells (PBMC) contributes to persistent lymphocyte activation and autoantibody production in SLE. Indeed, in vitro as well as in vivo, the level of apoptosis of PBMC of lupus patients has been shown to be elevated [4-7]. It has been suggested that this is due to increased in vivo activation of PBMC in SLE resulting in increased activation induced cell death [5]. Whether the level of apoptosis in SLE patients is in balance with the increased lymphocyte activation is, however, not clear as many other factors than cell activation do influence apoptosis. For example, apoptosis inhibiting factors such as soluble Fas and antibodies against Fas ligand have been shown to be elevated in SLE patients [8-10]. On the other hand, apoptosis can be facilitated and initiated by the use of immunosuppressive medication [11]. Studies
dealing with apoptosis in SLE have shown conflicting results. In certain strains of mice it has been demonstrated that mutations in the Fas (CD95) receptor or its ligand results in defective Fas mediated apoptosis and induce autoimmune phenomena [12,13]. Although a lymphoproliferative syndrome with the production of autoantibodies resulting from a genetic defect in the Fas system has been described in a few patients [14-16], up till now a FasL mutation has been described in SLE patients only incidentally. Common defects do not seem to occur[17]. Abnormalities in Fas-induced apoptosis have not been demonstrated [18]. Next to Fas signalling, induction of apoptosis can occur by signalling via the CD3 receptor. Kovacs et al. demonstrated defective induction of apoptosis by anti-CD3 in short term T (CD8+) cell lines of SLE patients [19]. The conflicting results concerning apoptosis induction in SLE between study populations can be accounted for by differences in disease activity, use of medication, or other exogenous factors influencing apoptosis, such as smoking habits. Therefore, to avoid the presence of these confounding factors, we tested whether anti-CD3 induced or anti-Fas-induced apoptosis are altered in inactive, non-smoking lupus patients using no or minor amounts of corticosteroids only.

**Patients and Methods**

**Patients and blood samples**

Patients eligible for this study fulfilled at least 4 American College of Rheumatology (ACR) criteria for SLE [20], were non-smokers, and had inactive disease. Inactive disease was defined as the persistent absence of clinical disease activity with a maximum SLEDAI score [21] of 4 for at least a 4 month period prior to blood sampling. Patients were allowed to use a constant dose of maximum 5 mg prednisolone a day. Patients using other immunomodulating drugs were excluded. Tobacco smoking was an exclusion criterium for this study as it augments Fas expression and thereby, probably, influences the susceptibility for apoptosis induction (manuscript submitted). The SLEDAI score was calculated for each patient. Fourteen healthy non-smoking volunteers, 3 males and 11 females, median age 27 years (range 22-39), were included as controls. For measurement of complement C3 and C4, and detection of anti-dsDNA antibody levels EDTA blood was drawn. Heparinized blood (30 ml) was drawn for cell isolation and culture.

**Measurement of complement, C-reactive protein, and detection of antibodies to double-stranded DNA**

Complement C3 and C4 were measured by nephelometry (normal values 0.64-1.20 g/l and 0.11-0.40 g/l, respectively). Serum levels of C-reactive protein, normal value <3 mg/l, were measured as previously described [22]. Anti-dsDNA antibodies were detected by Farr-assay using 125I-labeled recombinant ds-DNA (Diagnostic Products Corporation, Los Angeles, USA) which is free of contamination with ssDNA. Farr assay was performed according to the manufacturer's instruction and positive samples were measured at
different dilutions to obtain measurements within the assay range. Results of this assay were expressed in IU/ml using Wo/80 as the ultimate standard [23]. Normal value of this Farr-assay in our laboratory is <10 IU/ml; intra- and interassay variations are both < 10 %.

**Study design**

Cells were stained to measure Fas expression and the level of apoptosis immediately after isolation (t0). To obtain optimal Fas expression, cells were cultured for different time intervals under different circumstances: cells were incubated with or without 5 µg/ml anti-CD3 to evaluate stimulation via the CD3-receptor. IL-2 (100 IU/ml) was added to evaluate whether IL-2 deprivation could influence apoptosis results. Optimal up-regulation of Fas was achieved after 48 hours incubation with anti-CD3 (data not shown). Furthermore, the addition of IL-2 did not alter the level of apoptotic lymphocytes reached, indicating that in our culture system sufficient IL-2 was present (data not shown). Therefore, in all further stimulation experiments cells were cultured for 48 hours with anti-CD3 only. After 48 hours incubation with anti-CD3 (t1), Fas expression and level of apoptosis was measured. Next, anti-Fas or medium was added and after a further 24 hrs (t2) and 48 hrs (t3) Fas expression and levels of apoptosis were detected again.

**Cell isolation and culture**

Within 60 minutes after blood drawing peripheral blood mononuclear cells (PBMC) were isolated by lymphoprep density gradient centrifugation under sterile conditions. Cells were resuspended in medium (RPMI 1640 + 2 mM glutamin + 60 µg/ml gentamycin + 5 % human poolserum) at a concentration of 0.5 x 10^6/ml with 5 µg/ml anti-CD3 (mAb WT32). All cultures were performed in a culture flask at 37°C in a humidified atmosphere containing 5% CO₂. After 48 hours incubation with anti-CD3 (t1) cells were washed twice with RPMI 1640 and resuspended in medium with and without the addition of 5 µg/ml anti-Fas (CLB-CD95/15), kindly provided by dr. L. Aarden, CLB, Laboratory of Experimental and Clinical Immunology, Amsterdam, The Netherlands). Subsequently, cells were cultured for another 24 hours (t2) and 48 hours (t3). At all time points indicated part of the cells was used for apoptosis assessment as described below.

**Staining procedures, FACS analysis and apoptosis assays**

For staining we used the following conjugated monoclonal antibodies (MoAbs): anti-CD4 (allophycocyanine, APC) and anti-Fas/CD95 (phycoerythrin, PE), (Becton Dickinson, Mountain View, CA). Apoptosis was detected using fluorescein isothiocyanate (FITC) labeled annexin V, (Nexins, Hoeven, The Netherlands) and propidiumiodide (Molecular Probes, Leiden, The Netherlands). Apoptosis was defined as positive annexin V staining of the subpopulation of lymphocytes analyzed [24]. Annexin V binds to phosphatidylyserine which is exposed at the outer surface of the cell membrane already at early stages of apoptosis and remains so during the subsequent process of apoptosis. By defining apoptotic cells as those cells staining with annexin V, irrespective of
propidium iodide staining, we were able to detect early as well as late apoptotic cells. After washing with phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA), staining of $10^6$ cells was done by adding labelled MoAb with subsequent incubation for 45 minutes in the dark on ice. Samples were washed with PBS/BSA. After the addition of 222.5 µl binding buffer (10 mM hepes, pH=7.4/140 mM NaCl/2.5 mM CaCl$_2$) 25 µl propidium iodide (10 µg/ml) and 2.5 µl annexin V diluted 1:50 were added [24]. Immediately after incubation for 10 minutes in the dark on ice, three-color immunofluorescence analysis was performed on a Coulter Epics-Elite equipped with a gated amplifier (Coulter Electronics, Mijdrecht, The Netherlands). Cells were gated for lymphocyte characteristics using both forward and sideward scatter. All studies were performed with gates set for the total number of lymphocytes and for CD4$^+$ lymphocytes separately. As control for the level of apoptosis DNA content of cells was measured by the method of Nicoletti [25]. Apoptosis was defined as the percentage of cells with a fractional DNA content less than that of intact G1 cells (subdiploid peak). To exclude cellular debris, fragments with the 20% lowest DNA content were not included in the results [26]. In brief, $10^6$ cells were washed (PBS/BSA) and resuspended in 0.3 ml hypotonic propidium iodide solution (50 µg/ml) with 0.1 % sodium citrate (Janssen, Tilburg, The Netherlands) plus 0.1 % triton X-100 (Sigma, St Louis, USA) and 0.1 µg/ml RNase-A (Sigma, St Louis, USA). Analysis of DNA content was performed by flowcytometry.

Statistical analysis
Differences between groups were evaluated with the two-tailed Mann-Whitney U-test. Paired samples were analysed by Wilcoxon matched pairs signed ranks test. P< 0.05 was considered statistically significant.

Results

Thirteen SLE patients, 11 female and two male, were included. Their median age was 33 years (range 22-76) with a disease duration of median 9 years (range 0-21). Cumulative ACR criteria of the patients are shown in table 1. Two patients were on a low maintenance dose of prednisolone (one was on 5 mg/day, the other on an alternating scheme of 2.5/5.0 mg/day). All patients were clinically quiescent: 9 patients had a SLEDAI-score of 0; 3 patients had a SLEDAI-score of 2, which in one patient was due to slightly increased levels of antibodies against dsDNA (13 IU/ml) and in the others to decreased C4 levels (0.06 and 0.08 g/l, respectively). The remaining patient had a SLEDAI score of 4 due to a decreased level of complement C3 (0.58 g/l) and increased levels of antibodies against dsDNA (19 IU/ml). Levels of C3 and C4 were 0.78 g/l (0.58-1.04) and 0.19 g/l (0.06-0.40), respectively, for the total group of patients. None of the patients showed signs of infection. All patients had a C-reactive protein level < 3 mg/l.
Spontaneous and in vitro expression of Fas

Percentages of freshly isolated CD4⁺ lymphocytes positively staining for Fas did not differ between healthy controls and SLE patients (figure 1a). Mean fluorescence intensity (MFI) of Fas positive cells was higher in SLE patients (p=0.002, figure 1b). Incubation with anti-CD3 resulted in an increase of Fas positive CD4⁺ lymphocytes after 48 hours (t1, figure 1a). In controls the percentage of Fas-expressing CD4⁺ lymphocytes after CD3 stimulation was higher compared to SLE patients (figure 1a). Also, MFI of Fas-positive cells in SLE patients and controls was significantly higher after incubation with anti-CD3 than at baseline (figure 1b). After 48 hours incubation with anti-CD3 (t1), cells were washed, suspended in medium with or without anti-Fas, and cultured for another 24 (t2) or 48 (t3) hours. Culturing for another 24 hours without anti-Fas did not change Fas expression in SLE patients nor in controls (figure 1 a,b, t2 versus t1). In contrast, the addition of anti-Fas resulted in a decrease in the percentage of Fas positive cells and in the MFI of Fas positive cells; this decrease was similar in patients and controls (figure 1 a,b, t2a versus t2). Analysis of the total lymphocyte population for Fas expression showed results comparable to that of the CD4⁺ population for all experiments mentioned before.

Table 1:

<table>
<thead>
<tr>
<th>Cumulative ACR criteria</th>
<th>n</th>
<th>(%)</th>
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<tbody>
<tr>
<td>1. malar rash</td>
<td>5</td>
<td>(38)</td>
</tr>
<tr>
<td>2. discoid rash</td>
<td>4</td>
<td>(31)</td>
</tr>
<tr>
<td>3. photosensitivity</td>
<td>9</td>
<td>(69)</td>
</tr>
<tr>
<td>4. oral ulcers</td>
<td>2</td>
<td>(15)</td>
</tr>
<tr>
<td>5. arthritis</td>
<td>6</td>
<td>(46)</td>
</tr>
<tr>
<td>6. serositis</td>
<td>2</td>
<td>(15)</td>
</tr>
<tr>
<td>7. renal involvement</td>
<td>5</td>
<td>(38)</td>
</tr>
<tr>
<td>8. cerebral involvement</td>
<td>2</td>
<td>(15)</td>
</tr>
<tr>
<td>9. hematologic abnormalities</td>
<td>7</td>
<td>(54)</td>
</tr>
<tr>
<td>10. immunologic abnormalities</td>
<td>9</td>
<td>(69)</td>
</tr>
<tr>
<td>11. ANA</td>
<td>13</td>
<td>(100)</td>
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Apoptosis

Apoptosis of CD4⁺ lymphocytes was detected using annexin V staining. Percentages of apoptotic lymphocytes were detected by annexin V staining as well as by measuring the subdiploid peak using propidium iodide. Neither the percentages of apoptotic CD4⁺
lymphocytes nor that of total lymphocytes differed between SLE patients and controls immediately after lymphocyte isolation (t0, figure 2, 3a).

After activation with anti-CD3 (t1) levels of apoptotic T lymphocytes as well as levels of apoptotic CD4+ T cells significantly increased both in SLE patients and in controls. After anti-CD3 stimulation the percentage CD4+ apoptotic lymphocytes tended to increase in SLE patients (p=0.09 versus controls, figure 2) reaching significance for the total lymphocyte populations (p=0.03, figure 3a). Similar results were obtained after further incubation in medium (t2) or with anti-Fas antibodies (t2a), showing increased apoptosis of total lymphocytes in SLE patients compared to controls in both assays for apoptosis detection used (figure 3a,b). The increase in the percentage of apoptotic cells after incubation with anti-Fas was significantly higher compared to the control experiment in which cells were incubated in medium only. As expected, increase in annexin V staining occurred earlier in time compared to increase in the propidium iodide staining. After incubation with anti-Fas a peak in annexin V binding cells could be observed after 24 hours (t2), whereas propidium iodide staining peaked after 48 hours (t3, figure 3 a,b).

**Figure 1**: Percentages of Fas (CD95) positive lymphocytes (a) and mean fluorescence intensity (MFI) of these Fas positive lymphocytes (b) from controls (open bars) and SLE patients (black bars) at: t0, immediately after lymphocyte isolation; t1, after 48 hours incubation with anti-CD3; t2, after another 24 hours incubation in medium or, t2a, following incubation with anti-Fas antibodies. Results are expressed as mean ± SD. *p<0.05, **p<0.01, patients compared to controls, ***p<0.001 comparing different time points of evaluation.
Discussion

In this study we demonstrate that the percentage of apoptotic lymphocytes in peripheral blood in clinically quiescent SLE patients is unaltered compared to healthy controls although the MFI of Fas-positive lymphocytes is increased. Furthermore, the increase in the percentage of apoptotic lymphocytes after in vitro apoptosis induction with anti-CD3 and anti-Fas demonstrates that there are no defects in either anti-CD3 or anti-Fas induced apoptosis in SLE patients. In contrast, we found increased levels of apoptotic lymphocytes in SLE patients after stimulation with anti-CD3 as well as after 24 h incubation with anti-Fas compared with controls. Thus, we show increased levels of \textit{in vitro} apoptosis in SLE in a patient population with inactive disease and without use of immunosuppressive medication, factors which might have influenced results from previous studies [4-7]. Although the number of patients studied was limited, patients included in this study were a representative selection of our total population of SLE patients as cumulative ACR criteria in this group were similar to those of our whole cohort [27].

![Figure 2: Percentages of apoptotic CD4$^+$ lymphocytes from controls (open bars) and SLE patients (black bars) at: t0, immediately after lymphocyte isolation; t1, after 48 hours incubation with anti-CD3; t2, after another 24 hours incubation in medium or, t2a, in the presence of anti-Fas antibodies. Apoptosis was assessed by positive staining with annexin V. Results are expressed as mean ± SD. *p<0.05, **p<0.01, ***p<0.001 comparing different time points of evaluation.](image)

Alterations in Fas and FasL expression and function resulting in defective apoptosis as found in murine autoimmune mouse strains [12,13] have only incidentally been detected in human SLE [17]. Nevertheless, changes in the expression of apoptosis-related molecules and levels of apoptosis have been demonstrated in SLE. Fas expression has been reported to be elevated in SLE patients [5,18]. As Fas is up-regulated upon \textit{in vitro} cell activation [18,28,29], we carefully excluded such confounding factors in the present
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study. In our patients, we isolated PBL within one hour after blood sampling. We found the percentage of Fas positive, CD4⁺ lymphocytes and total lymphocytes to be similar compared with controls. MFI of Fas on freshly isolated lymphocytes from patients was, however, increased compared with controls, indicating a higher membrane expression for Fas on positively staining cells in SLE than in controls. This is probably due to the increased state of lymphocyte activation which can be found in vivo, even in SLE patients with inactive disease [1]. Upon stimulation with anti-CD3, Fas expression increased in controls and patients, in concordance with other studies [18]. The percentage Fas-expressing lymphocytes after anti-CD3 stimulation was somewhat lower in SLE patients which probably reflects a loss of in vivo preactivated lymphocytes. After incubation with anti-Fas antibodies Fas expression diminished, most likely due to the binding of these antibodies to their targets on the cell surface thereby inducing apoptosis. As apoptosis is a continuous process we choose to detect apoptotic cells by two different methods so minimising the influence of different detection techniques on the results.

Figure 3: (a) Percentages of apoptotic cells of the total lymphocyte population from controls (open bars) and SLE patients (black bars) at: t0, immediately after lymphocyte isolation; t1, after 48 hours incubation with anti-CD3; t2, after another 24 hours incubation in medium or, t2a, in the presence of anti-Fas antibodies. Apoptosis was assessed by positive staining with annexin V. Results are expressed as mean ± SD. *p<0.05, patients compared to controls; **p<0.01, ***p<0.001 comparing different time points of evaluation. (b) Percentages of apoptotic cells of the total lymphocyte population from controls and SLE patients at the same time points. The percentage apoptotic cells was defined as the percentage lymphocytes of the total lymphocyte population containing decreased levels of DNA (subdiploid peak). Results are extended with measurement after 48 hours incubation in medium or in the presence of anti-Fas antibodies (t3 and t3a respectively).
By measuring the DNA content with propidiumiodide late apoptotic cells were detected and by measuring annexin V binding early as well as late phases of apoptosis could be visualised. In freshly isolated peripheral blood cells we analysed the percentage of apoptotic CD4$^+$ and total lymphocytes. Neither method detected differences in the percentage of apoptotic lymphocytes between patients and controls, despite an increased MFI of Fas on isolated lymphocytes in SLE patients. Comparing MFI of Fas staining on freshly isolated lymphocytes from inactive SLE patients with that on lymphocytes after stimulation with anti-CD3, the former MFI proved rather low, suggesting a minor in vivo state of activation only that did not result in significant apoptosis. However, as we show in our induction experiments, the higher initial Fas expression in SLE might explain the higher percentages of apoptotic lymphocytes we found upon further activation with anti-CD3 in patients compared with controls. A contributing factor which leads to increased levels of apoptotic cells might have been a decreased clearance which has been described in SLE patients [30].

In agreement with Lorenz et al. we did not find increased levels of apoptosis of freshly isolated lymphocytes in SLE patients [5]. Others demonstrated increased levels of apoptotic peripheral blood mononuclear cells in SLE [4,6,7]. This controversy might be explained by differences in methodology. Lymphocytes of SLE patients may be preactivated in vivo. Therefore, any delay after blood sampling in detection of apoptosis will result in increased levels of apoptotic cells. We isolated lymphocytes and detected apoptosis within 1 h after blood sampling. Additional factors might explain the discrepancy of our results with those of others. As our study population had inactive disease and no or minor use of corticosteroids only, the results of our study are most likely representative for the in vivo situation in patients with quiescent SLE. In other studies patients were included irrespective of disease activity [4-7]. Although in these studies only a weak [4] or no [5-7] correlation between the level of apoptosis and disease activity was found, no conclusions can be drawn as data may have been influenced by the use of medication as well. Also differences in cell isolation and cell culture conditions can influence apoptosis [5]. For example, suboptimal concentrations of growth factors such as IL-2, can induce apoptosis. As addition of IL-2 did not influence levels of apoptosis, we feel that conditions were optimal in our study. In vitro activation-induced apoptosis in SLE patients did not seem to be disturbed. After stimulation with anti-CD3 apoptosis increased in controls and even more so in SLE patients. We, therefore, could not confirm defective CD3-mediated cell death of lymphocytes from SLE patients as reported by others [19]. In the latter study, based on experiments with T cell lines consisting predominantly of CD8$^+$ cells, it was found that activated T cells from patients with SLE were relatively resistant to anti-CD3 induced apoptosis. We analysed the CD4$^+$ and total T lymphocyte population. Neither in the whole T cell population nor in the CD4$^+$ subpopulation defects in anti-CD3 induced apoptosis could be found. A selective defect in activation induced cell death of CD8$^+$ lymphocytes can, however, not be excluded but seems unlikely.
Finally, we showed that anti-Fas induced apoptosis in SLE patients is functionally intact which confirms earlier studies [18,28]. We extend these data by showing that both activation-induced Fas expression and anti-Fas-induced apoptosis is normal in SLE patients with inactive disease.

In conclusion this study shows that in quiescent SLE patients, activation-induced and Fas-induced apoptosis are functionally intact. As in SLE patients Fas expression is increased on freshly isolated PBL, the slightly higher levels of apoptotic lymphocytes as seen after stimulation with anti-CD3 or anti-Fas might well represent minor changes due to preactivation of PBL of SLE patients in vivo.

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References


