Apoptosis and autoantibodies in systemic lupus erythematosus

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Chapter IV

Fas expression on peripheral blood lymphocytes in Systemic Lupus Erythematosus (SLE): relation to lymphocyte activation and disease activity

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Submitted
Summary

Levels of apoptotic lymphocytes have been found increased in SLE and persistence of apoptotic cells has been associated with autoantibody production. Increased lymphocyte Fas (CD95) expression due to lymphocyte activation may account for increased susceptibility for Fas-mediated apoptosis in SLE. Flow cytometry was performed to evaluate membrane expression of Fas in combination with the activation markers CD25, HLA-DR, and CD38 on, respectively, CD4+, CD8+, and CD19+ lymphocytes of SLE patients with inactive (n=20) and with active disease (n=13). SLEDAI-scores were calculated. Healthy volunteers (n=14) served as controls. Percentages of CD4+ T-cells expressing CD25 and CD19+ B-cells expressing CD38 were increased in patients with active disease compared to controls (p=0.03, p=0.04, respectively). In contrast to CD4+ and CD8+ cells, percentages of CD19+ cells expressing Fas were increased in SLE patients with active disease (p=0.0002, versus controls). In these patients percentages of cells double positive for both CD38 and Fas were increased compared to patients with inactive disease (p=0.006) and controls (p=0.0007). Percentages of CD19+ cells expressing Fas correlated with SLEDAI-scores.

In SLE patients, percentages of Fas expressing B-lymphocytes are increased, are related to the state of lymphocyte activation, and correlate to disease activity. We suggest that increased Fas expression results in a higher susceptibility for Fas mediated apoptosis. This might contribute to the increased levels of apoptotic lymphocytes in SLE patients.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterised by the presence of autoantibodies to nuclear and cytoplasmic antigens. The etiopathogenesis of the disease is unknown. Recently, a possible role of apoptotic cells in the pathogenesis of SLE has been suggested [1]. First, it has been demonstrated that during the apoptotic process autoantigens are exposed at the outer surface of the apoptotic cell. This might allow the development of autoantibodies directed to intracellularly localised antigens [2]. Secondly, injection of large numbers of apoptotic cells in animal models was shown to induce autoimmunity [3]. Indeed, in SLE patients increased levels of apoptotic lymphocytes have been detected in the peripheral blood [4]. Why these cells are present in the circulation is presently unknown. One explanation might be that phagocytosis of apoptotic cells is impaired in SLE patients [5]. Alternatively, or in conjunction, the production of apoptotic cells might be increased in lupus patients leading to an overflow of the phagocytic capacity. It has been suggested that the increased production of apoptotic cells results from increased lymphocyte activation [6] as is present in SLE patients even during clinically inactive disease [7,8]. The major route for induction of apoptosis in activated lymphocytes is mediated by Fas. Fas, also known as APO-1 or
CD95, is a cell surface protein of 45-48 kD belonging to the tumour necrosis factor (TNF)/nerve growth factor (NGF) receptor family [9,10]. Cross-linking of Fas by Fas ligand (FasL), a membrane glycoprotein of 40 kD, triggers apoptotic cell death with characteristic cytoplasmic and nuclear condensation and DNA fragmentation [11]. Fas-mediated apoptosis is functionally intact in SLE patients in vitro [12,13]. We wondered whether the increased levels of apoptotic lymphocytes in vivo could be attributed to increased lymphocyte activation concurrent with increased Fas expression in vivo. To elucidate these questions Fas membrane expression was measured on peripheral blood lymphocyte subpopulations in lupus patients in comparison to healthy controls, and results were related to the state of lymphocyte activation and disease activity.

Patients and Methods

Patients and blood samples
Patients eligible for this study fulfilled at least 4 American College of Rheumatology (ACR) criteria for SLE [14], were non-smokers, and had either active or inactive disease. Patients with active disease had to fulfil criteria as shown in table 1. Inactive disease was defined as the persistent absence of clinical disease activity for at least a 4 month period while patients were without or on a constant dose of immunomodulating drugs. To evaluate changes due to disease activity, reevaluation of patients with initially active disease took place at a moment of inactive disease as well. The SLEDAI score was calculated for each patient [15]. Healthy non-smoking volunteers, matched for age and sex, were included as control.

Blood samples for anti-dsDNA detection were drawn in EDTA and were subsequently analysed as described [16]. Heparinized blood was drawn for lymphocyte subset analysis. Samples were stained and analysed the same day by flow cytometry.

Cell isolation and staining procedure
Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by lymphoprep density gradient centrifugation. For surface staining the following phycoerythrin (PE) or fluorescein isothiocyanate (FITC) and allophycocyanine (APC) conjugated monoclonal antibodies (mAb) were used: CD4 (APC), CD19 (APC), CD25 (FITC/PE), CD38 (FITC/PE), HLA-DR (FITC/PE) and CD95 (PE) (Becton Dickinson, Mountain View, CA); CD8 (APC) (Pharmingen, Hamburg, Germany). After washing with phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA) staining of $10^6$ cells was done by adding labelled mAb with subsequent incubation for 45 minutes on ice in the dark. Samples were washed with PBS/BSA. Three-colour 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. For final analysis only those samples were included in which more than 500 cells of the respective subsets were counted.

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 (<50x10^9 /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 (>50x10^9/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

Statistics
Differences between groups were evaluated using the student’s t-test or Mann-Whitney U test when appropriate. Paired samples were analysed separately using the Wilcoxon rank
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sum test. Spearman’s rank correlation was applied for detecting correlations between different study parameters. A p value less than 0.05 was considered statistically significant.

Results

Twenty SLE patients were studied during inactive disease and 13 patients at a moment of active disease. Fourteen healthy volunteers served as control. Characteristics of the patients are given in table 2.

Table 2:

<table>
<thead>
<tr>
<th>Characteristics of patients with Systemic Lupus Erythematosus</th>
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<tr>
<td></td>
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<tr>
<td>Male/Female</td>
</tr>
<tr>
<td>(n=20)</td>
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<tr>
<td>(n=13)</td>
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<tr>
<td>3/17</td>
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<tr>
<td>1/12</td>
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<tr>
<td>age (years, mean, range)</td>
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<tr>
<td>43.4 (21-75)</td>
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<tr>
<td>39.2 (20-74)</td>
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<tr>
<td>Disease duration (years, mean, range)</td>
</tr>
<tr>
<td>10.9 (1-38)</td>
</tr>
<tr>
<td>7.5 (0-20)</td>
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<tr>
<td>SLEDAI (mean ± SEM)</td>
</tr>
<tr>
<td>2.6 ± 0.5</td>
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<tr>
<td>10.7 ± 1.4</td>
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<tr>
<td>Farr (IU/ml, mean ± SEM)</td>
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<tr>
<td>44.7 ± 13.1</td>
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<tr>
<td>149.2 ± 67.0</td>
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<tr>
<td>minor/major exacerbation</td>
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<td>-</td>
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<tr>
<td>7/6</td>
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<tr>
<td>renal disease</td>
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<td>-</td>
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<tr>
<td>5</td>
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<tr>
<td>central nervous system disease</td>
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<tr>
<td>-</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>Serositis</td>
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<td>-</td>
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<tr>
<td>3</td>
</tr>
<tr>
<td>Hematological disease</td>
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<td>-</td>
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<tr>
<td>5</td>
</tr>
<tr>
<td>Polyarthritis</td>
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<tr>
<td>-</td>
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<tr>
<td>4</td>
</tr>
<tr>
<td>skin involvement</td>
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<td>-</td>
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<td>2</td>
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<td>fever</td>
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<td>3</td>
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</tbody>
</table>

CD4+ T-lymphocytes of SLE patients with active disease showed signs of increased activation reflected by increased expression of CD25, the interleukin-2 receptor (p=0.03 versus controls, p=0.01 versus patients with inactive disease, figure 1a). Percentages CD8+ T-lymphocytes expressing the activation marker HLA-DR tended to be higher in patients with active disease (p=0.08 versus controls). Percentages CD19+ B-lymphocytes expressing the activation marker CD38 were increased in patients with active disease compared to controls (p=0.04, figure 1c). Fas expression was measured on CD4+ T-lymphocytes did not show significant differences between patients and controls, irrespective of disease activity.
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The percentage of Fas expressing CD8\(^+\) T-lymphocytes tended to be increased in patients with active disease (\(p=0.06\) versus controls, figure 2). However, the percentage of Fas expressing CD19\(^+\) B-lymphocytes was strongly increased in patients with active disease (\(p=0.0002\), versus controls). Also, patients with active disease showed a higher percentage of Fas expressing B-lymphocytes than patients with inactive disease (\(p=0.02\)).

To investigate whether Fas expression was associated with lymphocyte activation we analysed Fas expression in conjunction with CD38 expression as a membrane marker for B cell activation and related the data to disease activity. In SLE patients with active disease the percentage lymphocytes double positive for CD38 and Fas was significantly increased compared to patients with inactive disease (\(p=0.006\)) as well as compared to controls (\(p=0.0007\), figure 3). Consequently, lymphocytes double negative for these markers were significantly decreased in SLE patients with active disease (\(p=0.0001\) versus controls, \(p=0.01\) versus patients with inactive disease). Furthermore, when we analysed the relation between Fas expression and disease activity as measured by the SLEDAI-score a significant correlation between the percentage of Fas expressing CD19\(^+\) B cells and disease activity was found (figure 4). No correlation was found between disease activity and the percentages Fas expressing CD4\(^+\)- or CD8\(^-\)-T-lymphocytes.

**Figure 1**: Percentages of activated lymphocytes of the subpopulations analysed in controls and SLE patients. A: percentage of CD4\(^+\) cells expressing the activation marker CD25. B: percentage of CD8\(^+\) cells expressing the activation marker HLA-DR. C: percentage of CD19\(^+\) cells expressing the activation marker CD38. Horizontal lines denote the median. Only results of those samples are shown in which the number of cells measured exceeded 500.
Discussion

In this study we found an increased percentage of Fas expressing B-lymphocytes in SLE patients in vivo. This confirms in vitro results in which it was shown that Fas is properly upregulated upon activation in SLE [12,13] and it extends these findings to the *in vivo* situation. In addition, using three-colour flowcytometry, we showed that Fas expression on B-lymphocytes is related to the state of activation of these cells as well as to disease activity.

The relation between Fas expression, lymphocyte activation, and apoptosis is very complex, in SLE patients in particular. Many factors have been identified which potentially influence the interrelation of the factors mentioned. For example, antibodies to FasL and to poly (ADP-ribose) polymerase (PARP) have been found in SLE patients both of which may inhibit apoptosis [17,18]. Elevated levels of sFas in the serum of SLE patients [8,19-23] also can inhibit the induction of Fas-mediated apoptosis. Furthermore, the use of medication can influence the data obtained, especially since many immunosuppressives which are used in the treatment of SLE, such as corticosteroids and azathioprine, induce apoptosis thereby indirectly changing the characteristics of the circulating cell populations left [24].

B cell activation, resulting in the production of autoantibodies, plays a central role in the pathogenesis of SLE. In previous reports we showed that, prior to a relapse, an oligoclonal expansion of B cells capable of spontaneously

**Figure 2:** Fas (CD95) expression on CD4+- (panel A), CD8+- (panel B) and CD19+-lymphocytes (panel C) in controls and SLE patients with inactive and active disease. Horizontal line denotes the median. Only results of those samples are shown in which the number of cells measured exceeded 500.
producing anti-dsDNA in vitro can be demonstrated [7].

Recently, the dominant primary role of B cells has been demonstrated in the MRL/+ Fas intact mice model [25]. In this model, a B cell-deficient strain was created. Mice from this strain, in contrast to the wild type MRL/+ mice with functionally intact B cells, did not develop nephritis or vasculitis. In line with this role for B cells we showed that in SLE patients, during disease activity, indicators of lymphocyte activation are most pronounced for B cells. In addition, we demonstrated that upregulation of Fas occurs in conjunction with this B cell activation.

We could not demonstrate a significant relation between membrane Fas expression and the state of activation for the T lymphocyte subpopulations analysed. However, a tendency of increased Fas expression on CD8⁺-subpopulations of T-lymphocytes was present in patients with active disease. This is consistent with the results of others reporting increased expression of Fas on the total lymphocyte population [26] as well as on T cells [27]. Furthermore, also among CD4⁺- and CD8⁻-subpopulations of T-lymphocytes increased percentages of Fas positive cells have been described [12,28].

\[\text{Figure 3: Fas (CD95) expression on CD19⁺ B-lymphocytes in relation to the expression of the activation marker CD38 in controls and SLE patients with inactive and active disease. Horizontal lines denote the median.}\]
We propose that, in SLE, Fas membrane expression increases upon cell activation which renders peripheral blood lymphocytes susceptible for the induction of Fas-mediated apoptosis.

As it has been demonstrated that the Fas pathway is functionally intact in SLE [12, 13], upregulation of membrane Fas probably will result in increased induction of apoptotic lymphocytes after engagement of the Fas receptor. This will contribute to, or even cause, lymphopenia, which is present in many SLE patients and aggravates during disease activity. Indeed, Amasaki et al. found an inverse relation between Fas antigen intensity on CD4+ lymphocytes and absolute numbers of these cells [28].

A factor contributing to the presence of apoptotic cells might be defective clearance of these cells [5]. Together, increased induction of apoptosis and a decreased clearance capacity will result in the accumulation of apoptotic cells. During the process of apoptosis cells present nuclear antigens on their surface whether or not in modified form [2]. Continuous overpresentation of these antigens through the accumulation of apoptotic cells might break tolerance resulting in the autoimmune phenomena that characterise SLE or may boost an already existing autoimmune response.

In conclusion, we demonstrate in this study that the percentage peripheral blood B lymphocytes (CD19+) expressing Fas is increased in SLE patients and is related to the state of lymphocyte activation and the extent of disease activity. These data underscore the central role of B-lymphocytes in the pathogenesis of SLE. In addition, the data are compatible with the concept that upregulation of membrane Fas renders lymphocytes more susceptible for apoptosis resulting in increased rates of apoptotic PBL as described in SLE. Persistence of these apoptotic cells may play a primary role in the perpetuation of autoimmune responses.

**Figure 4:** Correlation between the percentage of Fas (CD95) expressing B (CD19+) cells and disease activity as measured by the SLEDAI-score. All moments of evaluation were included (n=33)

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16. ter Borg EJ, Horst G, Hummel EJ, Limburg PC, Kallenberg CG. Measurement of increases in anti-double-stranded DNA antibody levels as a predictor of disease exacerbation in


