Combination of anti-early apoptotic cell autoantibodies and anti-SSA autoantibodies in lupus nephritis

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Abstract: The aim of this study was to investigate the association of anti-early apoptotic cell autoantibodies, anti-SSA, and anti-SSB with clinical features of lupus nephritis (LN). Multiparameter flow cytometry was used to determine early apoptotic cells and for measuring the simultaneous binding of annexin V, 7-AAD, and IgG from LN patients (n = 39). The association between clinical features of LN and autoantibodies against early apoptotic cells, and between the autoantibodies and anti-SSA and anti-SSB, were further investigated. Thirteen LN patients (33.3 %) were positive for autoantibodies against early apoptotic cells. The prevalence of anti-SSA and anti-SSB were similar in patients with anti-early apoptotic cell autoantibodies and those without (anti-SSA: 9/13 versus 15/26; anti-SSB: 3/13 versus 4/26). Anti-early apoptotic cell antibody-positive patients had lower C3 levels (0.34 ± 0.22) than the antibody-negative patients (0.47 ± 0.17, p = 0.059); and significantly higher anti-dsDNA levels (502.99 ± 275.48 versus 214.13 ± 229.29, p = 0.001). In univariate logistic regression analysis, the presence of anti-early apoptotic cell antibody could predict poor short-term prognosis (HR 7.500, 95 % CI: 1.210 - 46.504, p = 0.030), while patients who were double positive for anti-SSA and anti-early apoptotic cell antibody had significantly increased risk of poor short-term outcome (HR 17.500, 95 % CI: 2.500 - 122.500, p = 0.004). The combination of anti-early apoptotic cell autoantibodies and anti-SSA might be of predictive value in LN.

Key words: Lupus nephritis; Early apoptotic cells; Autoantibody; Anti-SSA anti-SSB.

Introduction

Systemic lupus erythematosus (SLE) is a prototypical autoimmune disease characterized by the production of multiple pathogenic autoantibodies associated with multiple organ damage. Lupus nephritis (LN) is a major contributor to morbidity and mortality in patients with SLE (1). Decades of investigations have revealed that a variety of potentially pathogenic autoantibodies are implicated in SLE. These are anti-double-stranded DNA (anti-dsDNA) antibodies (2-4), anti-C1q antibodies (5-8), anti-ribosomal P proteins antibodies (9), anti-Sm antibodies (10), and anti-C-reactive protein (CRP) antibodies (11-13). Although there is a consensus on the involvement of some of these autoantibodies in the etiology of SLE and/or LN, only few autoantibodies have been reported as predictors of clinical outcomes. Most interestingly, very little attention has been given to autoantibodies against SSA(Ro)/SSB(La) in the pathogenesis of SLE, unlike other antibodies such as anti-C1q and anti-dsDNA. However, high titer of anti-SSA antibodies have been found in eluates from kidneys of patients with progressive renal disease (14), and several clinical studies have demonstrated that the presence of anti-SSA or anti-SSB antibodies is correlated with poor outcome in LN (15, 16).

It has been suggested that apoptotic cells play important roles in the induction of autoimmunity in SLE (17-19). Studies have shown that IgG fractions from all SLE patients investigated were bound to late apoptotic cells, due probably to presence of numerous autoantigens exposed at the late stages of apoptosis (20). On the other hand, only a subset of SLE patients have autoantibodies that recognize early apoptotic cells, and SSA/Ro is the major autoantigen exposed on the surface of early apoptotic cells (21). In neonatal lupus syndrome (NLS), the recognition of anti-SSA/SSB by the antigens exposed on apoptotic cells is thought to be involved in the pathogenesis of cardiac injury (22, 23). Maternal anti-SSA/SSB autoantibodies transported across the placenta could bind to the surface of apoptotic fetal cardiocytes. This impairs the clearance of apoptotic fetal cardiocytes and promotes secretion of inflammatory cytokines, thereby resulting in tissue damage (24, 25). It is not yet clear whether recognition of early apoptotic cells is one of the mechanisms involved in anti-SSA/anti-SSB-induced pathological cascade of events in non-neonatal SLE, as it is in NLS.

Autoantibodies against early apoptotic cells have only been tested in anti-SSA positive SLE patients (21). It is still uncertain whether these autoantibodies also exist in anti-SSA-negative SLE patients. In the current study, the prevalence of autoantibodies against early apoptotic cells in a small cohort of Chinese patients with LN was investigated. In addition, the associations between anti-early apoptotic cell autoantibodies, anti-SSA, anti-SSB, and the clinical features of LN were determined.

Materials and Methods

Patients and sera

Blood samples from 39 patients with active LN who were admitted to our hospital between January 2013 and
August 2015 were collected separately into pro-coagulation tubes at presentation. The blood samples collected were then centrifuged at 3000 rpm for 10 min and the sera separated as such were stored at -80 °C until use. Among the 39 patients, 34 (87.2 %) were females and 5 (12.8 %) were males. The age of the patients ranged from 14 to 50 years, with a mean age of 34.0 ± 11.6 years. The patients fulfilled the 1997 American College of Rheumatology (ACR) revised criteria for SLE, and the diagnosis of LN was according to clinical and laboratory manifestations consistent with ACR criteria (26). The clinical disease activity was assessed using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (27). The criteria for clinical remission were the same as reported previously (28). Complete remission was marked by urinary protein excretion < 0.3 g/day, with normal urinary sediments (RBC < 3/ HP, WBC < 5/HP); serum albumin, and renal function. Partial remission was denoted by the presence of any of the following features: decreased serum creatinine to levels below 130 μmol/L, with a baseline serum creatinine ≥130 μmol/l, but ≤260 μmol/l; 50 % decrease in serum creatinine, with a baseline serum creatinine > 260 μmol/l; decrease in urinary protein excretion > 50 %, and below 3.0 g/24 h, with serum albumin ≥30 g/L and stable renal function (29). The patients were followed up for 1 year. The primary end point was defined as death with active renal disease, while the secondary end point was defined as end stage renal disease (ESRD) or doubling of serum creatinine.

The detailed clinical data of the patients were retrospectively reviewed. Informed consent was obtained for blood sampling. The research was in compliance of the Declaration of Helsinki, and ethical approval was obtained from hospital ethics committee of Shandong Provincial Hospital affiliated to Shandong University (No.2013-075).

Sera from 15 healthy subjects matched for gender and age were collected and used as normal controls. All sera were stored at -80 °C until use.

Assay of autoantibodies
Serum antinuclear antibodies (ANA) were assayed using indirect immunofluorescence assay (Euroimmun, Lübeck, Germany). Anti-double-stranded DNA (dsDNA) antibodies, anti-Sm, anti-SSA, and anti-SSB were determined using enzyme-linked immunosorbent assay (ELISA) (Euroimmun, Lübeck, Germany).

Preparation of IgG
Immunoglobulin G (IgG) was purified from patient sera and control sera on protein G-Sepharose columns (Pharmacia) according to the manufacturer’s recommendations. Briefly, serum IgG was bound to protein G columns, washed with 50-column volumes of phosphate buffered saline (PBS), and eluted with 0.1 M glycine (pH 2.7). The eluate was neutralized by collection in 2M Tris-HCl (pH 9.0) and dialyzed against PBS.

Induction of apoptosis
Jurkat cells were washed twice with serum-free RPMI 1640, and then resuspended at a concentration of 1×10⁶ cells/ml and irradiated with UVC light for 3 min. After UV irradiation, the cells were cultured for 3 h in serum-free RPMI medium so as to harvest the early apoptotic cells (30). Early apoptosis was confirmed by double-staining with fluorescein isothiocyanate (APC)–labeled annexin V and 7-AAD according to the instruction manual of Calbiochem (Darmstadt, Germany).

Binding assay
Immunoglobulin G (IgG) binding assay was performed according to a previously described method, but with mild modification (16). Apoptotic cells were washed twice in PBS, and 1 × 10⁶ cells were resuspended in 100 μl of 3 % BSA/PBS and incubated with 500 μg/ml of patient or control IgG at 4 °C for 1 h. The washing was repeated, and the cells were stained with 1:100 dilutions of secondary antibody, fluorescein isothiocyanate (FITC)-conjugated anti-human IgG (Abcam, Cambridge, USA) at 4 °C for 30 min. A combination of FITC-labeled anti-human IgG and APC-labeled annexin V/7-AAD was used to assess the early apoptotic cells that were bound to IgG.

Statistical analysis
The detailed clinical data of the patients were collected and used as normal controls. Statistical software SPSS 22.0 (SPSS, Chicago, IL, USA) was employed for statistical analysis. Quantitative data were expressed as mean ± standard deviation, (SD); median with range (minimum, maximum), or number and percentage (%). For comparison of clinical features of patients, t-tests, Mann-Whitney U test and chi square (χ²) test were used. Logistic regression model was applied for identifying prognostic factors associated with renal outcome. Only variables with p < 0.1 in the univariate logistic regression analysis were used in the multivariate logistic regression. Results were expressed as hazard ratio (HR) with 95 % confidence intervals (CI). Statistical significance was assumed at p < 0.05.

Results
Baseline data of the LN patients
As shown in Table 1, all the patients were positive for ANA; 28 patients (71.8 %) were anti-dsDNA positive, 43.6 % patients had anti-Sm antibody, and 61.5 % patients were positive for anti-SSA antibody. In the patients, anti-SSB antibody was always accompanied by anti-SSA antibody: 17.9 % patients were positive for both anti-SSA and anti-SSB.

In the study, 32 out of the 39 patients received 60 - 80 mg/day prednisone therapy, with 3 patients receiving a pulse of methylprednisolone at the beginning because of the presence of acute renal failure. Among the 32 patients, 27 patients completed treatment with monthly intravenous cyclophosphamide (800 - 1000 mg/month), while 5 patients received mycophenolate mofetil. After 1-year follow-up, all the 32 patients achieved clinical remission, 23 with complete remission and 9 with partial remission.

The 7 patients who were resistant to sequential treatment with pulse of methylprednisolone, intravenous cyclophosphamide, and mycophenolate mofetil, with or without plasmapheresis and intravenous immunoglobulins, reached the end points.

However, 4 of them died in the first 3 months due to renal failure (n = 3), and hemorrhage of digestive tract with continuous heavy proteinuria and severe hypoalbu-
Prevalence of autoantibodies against early apoptotic cells in LN patients

Multiparameter flow cytometry was utilized for selecting early apoptotic cells (annexin V positive, 7-AAD negative), and patient and control IgG were tested for reactivity to the apoptotic cells. The percentage binding of IgG from 15 healthy controls to early apoptotic cells were 5.08 ± 1.36%, and the cut-off value was 9.16% (3 SD above the mean percentage binding of healthy controls). As shown in Figure 1, 13 of the 39 patients (33.3%) were positive for autoantibodies against early apoptotic cells.

Associations of anti-early apoptotic cell autoantibodies with the presence of anti-SSA, anti-SSB, and the clinical features of LN

Among the 13 anti-early apoptotic cell antibody-positive patients, 9 were positive for anti-SSA antibody (3 of whom were double positive for anti-SSA and anti-SSB), while the other 4 patients were double negative for anti-SSA and anti-SSB. The prevalence of anti-SSA and anti-SSB was similar in patients with, and patients without anti-early apoptotic cell autoantibodies (9/13 versus 15/26 and 3/13 versus 4/26 for anti-SSA and anti-SSB, respectively). As shown in Table 2, anti-early apoptotic cell antibody-positive patients had lower C3 levels and significantly higher anti-dsDNA levels than anti-early apoptotic cell antibody-negative patients (C3 levels: 0.34 ± 0.22 versus 0.47 ± 0.17, p = 0.059; anti-dsDNA levels: 502.99 ± 275.48 versus 214.13 ± 229.29, p = 0.001).

Table 1. Baseline characteristics of the study patients with lupus nephritis.

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>39</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/female)</td>
<td>5/34</td>
</tr>
<tr>
<td>Age (mean ± SD.) (years)</td>
<td>34.0 ± 11.6</td>
</tr>
<tr>
<td>Number of anemia cases (%)</td>
<td>27 (69.2)</td>
</tr>
<tr>
<td>Number of leukocytopenia cases (%)</td>
<td>9 (23.1)</td>
</tr>
<tr>
<td>Number of thrombocytopenia cases (%)</td>
<td>14 (35.9)</td>
</tr>
<tr>
<td>Number of hematuria cases (%)</td>
<td>29 (74.4)</td>
</tr>
<tr>
<td>Number of leukocyturia (non-infection) cases (%)</td>
<td>28 (71.8)</td>
</tr>
<tr>
<td>Number of acute renal failure cases (%)</td>
<td>13 (33.3)</td>
</tr>
<tr>
<td>Hemoglobin (mean ± SD) (g/l)</td>
<td>96.1 ± 23.7</td>
</tr>
<tr>
<td>Urine protein (mean ± SD) (g/24 h)</td>
<td>4.0 ± 2.50</td>
</tr>
<tr>
<td>Serum creatinine (mean ± SD) (µmol/l)</td>
<td>113.3 ± 101.0</td>
</tr>
<tr>
<td>C3 (mean ± SD) (g/l)</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>SLEDAI (mean ± SD)</td>
<td>20.7 ± 5.5</td>
</tr>
<tr>
<td>ANA (%)</td>
<td>39 (100.0)</td>
</tr>
<tr>
<td>Anti-dsDNA antibody (%)</td>
<td>28 (71.8)</td>
</tr>
<tr>
<td>Anti-Sm (%)</td>
<td>17 (43.6)</td>
</tr>
<tr>
<td>Anti-SSA antibody (%)</td>
<td>24 (61.5)</td>
</tr>
<tr>
<td>Anti-SSB antibody (%)</td>
<td>7 (17.9)</td>
</tr>
</tbody>
</table>

*SLEDAI = SLE Disease Activity Index; ANA = antinuclear antibody; SSA = Sjögren’s syndrome A antigen; SSB = Sjögren’s syndrome B antigen.

Clinical association between the presence of anti-SSA and anti-early apoptotic cell autoantibodies

Since SSA is reported to be probably the preferential autoantigen expressed on the surface of early apoptotic cells (21), the clinical features of patients who were double positive for anti-SSA and anti-early apoptotic cell autoantibodies were investigated. As shown in Table 3, patients who were positive for the two antibodies had significantly lower C3 levels and higher anti-dsDNA levels than the other patients (C3 levels: 0.30 ± 0.12 versus 0.46 ± 0.20, p = 0.039; dsDNA levels: 507.17 ± 308.17 versus 247.48 ± 242.47, p = 0.012).

Renal outcome and the presence of anti-early apoptotic cell autoantibodies and anti-SSA autoantibodies

After 1-year follow-up, 32 patients achieved clinical remission, while 7 patients reached the end points. Baseline anti-early apoptotic cell antibody levels were

Figure 1. Binding of serum IgG to the surfaces of early apoptotic cells. A. Apoptotic cells were produced by exposure to UVC light for 3 min, and then cultured in serum-free RPMI 1640 for 3 h. 54.4% of the cells were early-apoptotic (annexin V positive/7-AAD negative) with very few late apoptotic cells (annexin V positive/7-AAD positive). B. Representative IgG binding to early apoptotic cell populations selected as annexin V positive, 7-AAD negative. Unfixed apoptotic cells were incubated with 500 µg/ml IgG, followed by fluorescein isothiocyanate–conjugated anti-human IgG. They were then stained with allophycocyanin (APC)–conjugated annexin V and 7-AAD. The dotted line represents blank control; the dashed line represents FITC-secondary antibody control, while the solid line is a representative positive IgG from a patient with LN. C. Binding of IgG from patients with LN and healthy controls to early apoptotic cells. Each dot represents the mean value of 3 to 5 independent experiments. Values above the dashed horizontal line (3 SD above the mean percentage binding of healthy controls) were considered positive.

Figure 2. Binding of IgG to early apoptotic cells from patients with good outcome and patients with poor outcome. At baseline, there was a highly significant difference in anti-early apoptotic cell antibody levels between patients with good outcome and patients with poor outcome, as shown by analysis with Mann-Whitney U test. The thick lines represent median values.
significantly higher in patients with poor short-term outcome ($p = 0.0019$; Figure 2).

Table 4 shows the results of univariate logistic regression analysis of 1-year prognosis of patients with LN. The results reveal that sex (male), SLEDAI, serum creatinine, anti-SSB antibody at baseline are risk factors. Furthermore, the presence of anti-early apoptotic cell antibody at baseline could predict short-term prognosis in this cohort (HR 7.500, 95% CI: 1.210 - 46.504, $p = 0.030$). Although anti-SSA was not correlated with prognosis due probably to the high prevalence of anti-SSA antibody in this cohort, patients who were double positive for anti-SSA and anti-early apoptotic cell antibody at baseline had a significantly increased risk of poor short-term outcome (HR 17.500, 95% CI: 2.500 - 122.500, $p = 0.004$). Moreover, in a further multivariate logistic regression analysis of the risk factors, combination of the two antibodies seemed to be a potential independent risk factor of poor short-term outcome ($p = 0.084$).

### Discussion

Results from assays of autoantibodies against early apoptotic cells in anti-SSA positive SLE patients have suggested that SSA is the major autoantigen exposed on the surface of early apoptotic cells (21). In that study (21), it was also found that anti-early apoptotic cell antibody existed in SLE patients with anti-SSA but was absent in those with both anti-SSA and anti-SSB. The current study is the first study to determine the prevalence of anti-early apoptotic cell autoantibodies in a LN cohort. The prevalence of anti-early apoptotic cell antibody in active LN was 33.3 %, and the prevalence...
of anti-SSA and anti-SSB was similar in patients with anti-early apoptotic cell autoantibodies and those without anti-early apoptotic cell autoantibodies. In other words, anti-early apoptotic cell antibody did not distinguish between patients with anti-SSA alone and those with both anti-SSA and anti-SSB in the cohort studied. The patients enrolled in this study were all renal SLE, which might be one explanation for the discrepancy between the results obtained in the present study and results from previous studies. Moreover, anti-early apoptotic cell antibody was also detected in LN patients with neither anti-SSA nor anti-SSB. Thus, there is a need to investigate whether autoantigens other than SSA are exposed on early apoptotic cells. However, even if there are other autoantigens existing on the surface of early apoptotic cells, SSA (Ro) appears to be the preferred antigen. More importantly, the pathogenic role of the recognition of anti-SSA/SSB by the antibodies exposed on apoptotic cells in NLS, as well as the predictive value of anti-SSA/anti-SSB ratio for ESRD in SLE patients have been suggested in some studies (15, 28). Furthermore, a recent study found that the Ro 60 autoantigen binds to endogenous retroelements and regulates inflammatory gene expression (31). This implies a pathologic role for anti-SSA antibodies in SLE. Therefore, by way of further studies, the associations of anti-early apoptotic cell autoantibodies, in combination with anti-SSA, with the clinical features and short-term outcomes of LN were investigated in the present study. It was found that anti-early apoptotic cell antibody, whether combined with anti-SSA or not, was not correlated with indices of disease activity such as proteinuria, serum creatinine, and SLEDAI scores, but were correlated with lower C3 levels and higher anti-dsDNA levels.

Lupus nephritis (LN) is a major cause of morbidity and mortality in patients with SLE. Although treatment with glucocorticoids and immunosuppressants has improved survival and renal outcomes in many LN many patients, these therapeutic strategies are not sufficient for controlling the disease in a significant proportion of patients. Indeed, their clinical prognosis remains poor due to development of ESRD or vital organ failure. In a recent study, it was shown that LN patients who were unresponsive to therapies in the first year were more than 10 times likely to be unresponsive during prolonged therapy (13). In the present study, 17.9% of patients were resistant to current therapies and reached the end points after 1-year follow up. In univariate logistic regression analysis, the risk factors for short-term outcome identified were sex (male), SLEDAI, serum creatinine, and anti-SSB antibody at baseline. Notwithstanding the smaller number of patients used, these findings are consistent with the results of a previous analysis of risk factors for long-term outcome in a large cohort of Chinese LN patients (28). More importantly, using univariate survival analysis, it was found that the presence of anti-early apoptotic cell antibody at baseline could predict short-term prognosis, and the predictive value was significantly strengthened by combination of anti-early apoptotic cell antibodies and anti-SSA antibodies. In multivariate logistic regression analysis, being double positive for anti-SSA and anti-early apoptotic cell antibody was not a significant independent predictor of short-term outcome. However, the very high hazard ratio value and borderline significance did not allow its rejection as an independent predictor of prognosis. Based on clinical evaluation and previous studies, it can be reasonably proposed that anti-SSA antibody probably contributes to the pathologic cascade of SLE via recognition to early apoptotic cells.

To the best of the knowledge of the authors, the present study is the first study to assess the predictive role of
anti-early apoptotic cell antibody combined with anti-SSA in short-term follow-up.

The relatively small sample size (accompanied by very low statistical power of used tests) is probably the major limitation of this study. As a result, the calculated ORs were not precise (95 % CIs were very wide), so that several interesting findings could not be considered as being significant. Another limitation of the study is that some of the patients did not receive renal biopsy due to apprehensions about postoperative complications of puncture or contradiction such as severe thrombocytopenia. Although the prognostic importance of renal biopsy in SLE has been the subject of much debate (32-35), the association between anti-early apoptotic cell autoantibodies and renal histological types, especially chronic lesions, vascular lesions and crescent lesions, need to be further investigated.

The results obtained in this study demonstrate that anti-early apoptotic cell antibodies, in combination with anti-SSA antibodies at baseline, might be strong predictors of poor short-term outcome in LN. However, this is just a tentative study, and the small sample size limits to some extent the generalization of the findings. Thus, a prospective study involving a larger sample size is needed to confirm these findings.

Acknowledgments
This study was supported by grants from the National Natural Science Foundation of China (No. 81300587).

Interest conflict
No competing interest is associated with this study. Informed consent was obtained for blood sampling.

Author’s contribution
All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Xiaowei Yang; Xiang Liu collected and analysed the data; Kamal Aktar wrote the text and all authors have read and approved the text prior to publication.

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