Elevated serum anti-endothelial cell autoantibodies titer is associated with lupus nephritis in patients with systemic lupus erythematosus

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Background and Purpose: Systemic lupus erythematosus (SLE) is an autoimmune connective tissue disease associated with endothelial dysfunction and the existence of multiple species of autoantibodies. However, the association between endothelial dysfunction and renal manifestations remains unclear in Taiwanese SLE patients.

Methods: Serum samples were collected from SLE patients with biopsy-proven lupus nephritis (n = 32), stable SLE patients (n = 32) and healthy controls (n = 32). The SLE Disease Activity Index (SLEDAI) of SLE patients was scored, and levels of anti-endothelial cell antibodies (AECA) and anti-endothelial activities in serum samples were measured by cell-enzyme-linked immunosorbent assay and crystal violet assay, respectively, using cultured human endothelial EA.hy926 cells.

Results: Significantly higher AECA (p<0.001) and anti-endothelial activities (p<0.001) were found in sera from patients with lupus nephritis compared with that from stable SLE patients or controls. Moreover, AECA titers (p<0.001) and anti-endothelial activities (p<0.001) were strongly correlated with SLEDAI scores in these patients.

Conclusion: The strong correlations of AECA and anti-endothelial activity with lupus nephritis activity support an endothelial origin for renal complications in Taiwanese SLE patients.

Key words: Anti-endothelial cell antibody; Endothelium; Lupus nephritis; Systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by involvement with a broad spectrum of clinical manifestations and the existence of multiple species of autoantibodies. The loss of immune tolerance, increased antigenic load, excess T cell help, defective B cell suppression, and the shifting of T helper 1 (Th1) to Th2 immune responses result in B cell hyperactivity and the production of pathogenic autoantibodies [1]. However, despite intensive investigation, the exact pathogenesis of SLE remains poorly understood [1]. Endothelial dysfunction is the predominant manifestation of SLE. Anti-endothelial cell antibodies (AECA) are a heterogeneous group of autoantibodies directed against different antigens in endothelial cells and have been identified in a variety of diseases including SLE, systemic vasculitis, systemic sclerosis and Kawasaki disease [2]. The proportion of AECA-positive sera ranges from 15% to 80% in SLE cases worldwide. However, the association of AECA levels with SLE disease activity, particularly lupus nephritis, remains to be delineated. In this study, we determined the AECA titer and the anti-endothelial activities in serum from SLE patients with active nephritis or stable disease, in order to evaluate possible correlation with clinical parameters of SLE. The strong correlation between AECA titer and disease activities of lupus nephritis suggests that AECA-mediated endothelial dysfunction may be involved in the pathogenesis of renal disorders in SLE.

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Methods

SLE patients
Sixty four SLE patients, who fulfilled the SLE criteria of the American College of Rheumatology, were enrolled at the Rheumatology Clinic at Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan. The clinical and laboratory parameters of SLE patients were recorded. The disease activities were scored according to SLE Disease Activity Index (SLEDAI). Lupus nephritis was verified by light microscopy, immunofluorescence, and electronic microscopy analysis. SLE patients were divided into two groups: active nephritis (32 SLE patients [29 females and 3 males] with biopsy-proven lupus nephritis including 24 cases of class IV, 6 cases of class V, 1 case of class III+V, and 1 case of class IV+V; SLEDAI score >6; age [mean ± standard deviation (SD)], 31.93 ± 10.6 years) and stable SLE (32 stable SLE patients [29 females and 3 males] with prednisolone dosage ≤5 mg/day; SLEDAI score ≤6, age [mean ± SD], 34.94 ± 9.3 years). The sera were collected from SLE patients and 32 controls (29 females and 3 males; mean age, 34.13 ± 6.8 years), then aliquoted and stored at −70°C until use. For SLE patients with active nephritis, sera were obtained prior to renal biopsy.

Assay for AECA titer
The AECA titer in sera of SLE patients were determined by cell-enzyme-linked immunosorbent assay (cell-ELISA) as previously described with slight modification [3]. EA.hy926 endothelial cells [4] were seeded onto 96-well microtiter plates (density, 10⁴ cells per well) and cultured with Dulbecco’s Modified Eagle’s Medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 mg/mL streptomycin and 100 U/mL penicillin, 1% hypoxanthine, aminopterin, and thymidine at 37°C and 5% carbon dioxide. After growth to near confluence, cells were washed with phosphate-buffered saline (PBS) and fixed with ethanol (100 µL per well) on ice for 5 min. After washing with PBS three times, cells were blocked with 2% bovine serum albumin for 1 h at 37°C. After washing with PBS three times, 100 µL of serum (1:100 diluted with PBS) was added into each well. After overnight incubation, cells were washed then incubated with horseradish peroxidase-conjugated anti-human immunoglobulin G (IgG; diluted 1:4000; Molecular Probe, OR, USA) for 1 h. The detection was performed by incubation with tetramethylbenzidine chromogen/substrate solution (Wampole Laboratories, Cranbury, NJ, USA) for 30 min then terminated by adding 50 µL stop solution to each well. For each serum sample, the absorbance at 492 nm in wells with EA.hy926 cells was subtracted from that in wells without cells and expressed as mean ± SD percentage increment over control from triplicate data. The absorbance at 492 nm of sera from three healthy volunteers was averaged and used as the control value (100%).

Assay for anti-endothelial activity
The anti-endothelial activities in serum of SLE patients were determined using crystal violet assay. Briefly, cultured endothelial EA.hy926 cells were harvested by trypsin-ethylenediamine tetra-acetic acid and seeded onto 96-well microtiter plates (at a density of 10⁴ cells per well), and 5 µL sera from SLE patients or controls was added into each well. After 24 h, cells were stained with 0.05% crystal violet solution (in 3.7% formalin in PBS; 30 µL per well) for 1 h, washed with distilled water three times and solubilized with solution containing 50% ethanol and 0.1% acetic acid. The optical density of viable cells was measured by reading absorbance at 595 nm using scanning multi-well spectrophotometer, and then expressed as mean ± SD percentages of inhibition from triplicate data. The absorbance at 595 nm of sera from three additional healthy volunteers was used as control (100%).

Statistical analysis
The experimental data were expressed as mean ± SD from indicated number of patients or experiments. Comparisons between groups of independent samples were assessed by Mann-Whitney U test. The correlation between variables was determined by Pearson’s correlation test. A p value <0.05 was considered statistically significant.

Results
We measured the clinical parameters, AECA levels and anti-endothelial activities in sera from 32 SLE patients with biopsy-proven lupus nephritis, 32 stable SLE patients, and 32 healthy controls. All of the active SLE patients with lupus nephritis had proteinuria, white blood cell/red blood cell sediments and variable granular and/or hyaline casts in urinalysis. In addition, thirteen patients had nephrotic syndrome. The mean ± SD renal biopy activity index from the 32 active SLE patients was 8.81 ± 4.84, and the chronicity index was 1.22 ± 1.93.
AECA titer and lupus nephritis

Analysis of serological factors revealed that patients with lupus nephritis had significantly lower levels of complement 3 (C3; \( p < 0.001 \)) and hemoglobin (Hb; \( p < 0.001 \)), but higher values of anti-double-stranded DNA (anti-dsDNA) titer (\( p < 0.001 \)), SLEDAI score (\( p < 0.001 \)) and creatinine level (\( p = 0.03 \)) than stable SLE patients (Table 1).

The AECA titer was significantly increased in patients with lupus nephritis (mean increment, 192 ± 163%) compared with that in stable SLE (68 ± 54%; \( p < 0.001 \)) or controls (–5.5 ± 26%; \( p < 0.001 \)) [Fig. 1].

Sera from patients with lupus nephritis exhibited higher inhibitory activities (mean inhibition, 16 ± 7%) compared with that from stable SLE (mean inhibition, 6.7 ± 7.9%; \( p < 0.001 \)) or controls (mean inhibition, 0.35 ± 6.12%; \( p < 0.001 \)) [Fig. 2].

The association of serum AECA titer or anti-endothelial activity with clinical parameters of SLE was analyzed by Pearson’s correlation. It was found that AECA titer was strongly correlated with the levels of C3 (\( p < 0.001 \)), C4 (\( p < 0.05 \)), anti-dsDNA (\( p < 0.001 \)), white blood cells (\( p < 0.05 \)), and Hb (\( p < 0.01 \)) and SLEDAI score (\( p < 0.001 \)) [Table 2]. Also, the anti-endothelial activities were significantly associated with the level of C3 (\( p < 0.003 \)), Hb (\( p < 0.001 \)) and SLEDAI score (\( p < 0.001 \)) in SLE patients. Thus, both AECA and anti-endothelial activities are elevated during the progression of lupus nephritis.

To investigate the link between anti-endothelial activities and the titer of anti-endothelial IgG autoantibodies, the relationship between AECA level and anti-endothelial activity was examined in different groups of SLE patients and controls (Fig. 3). Interestingly, in normal individuals, AECA titer was significantly correlated with the anti-endothelial activity (\( p = 0.021 \); \( r = 0.42 \)), suggesting that AECA might be the predominant inhibitory factor to endothelial proliferation in normal physiological conditions (Fig. 3C). In stable SLE patients, AECA titer remained marginally associated with anti-endothelial activity despite prior SLE onset or medication (\( p = 0.074 \); \( r = 0.321 \)) [Fig. 3B]. However, such correlation no longer existed in SLE patients with lupus nephritis (\( p > 0.05 \); \( r = -0.02 \); Fig. 3A).

### Table 1. Serologic and clinical parameters of systemic lupus erythematosus (SLE) patients with lupus nephritis or in remission

<table>
<thead>
<tr>
<th>Serologic parameter</th>
<th>SLE with active nephritis (( n = 32 ))</th>
<th>Stable SLE (( n = 32 ))</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement 3 (mg/dL)</td>
<td>45 ± 20</td>
<td>84 ± 19</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Complement 4 (mg/dL)</td>
<td>10 ± 6</td>
<td>22 ± 33</td>
<td>NS</td>
</tr>
<tr>
<td>Anti-double-stranded DNA (IU/mL)</td>
<td>180 ± 150</td>
<td>38 ± 41</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DPL (g/day)</td>
<td>5.9 ± 5.2</td>
<td>2.4 ± 0.6</td>
<td>ND</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>1.6 ± 1.4</td>
<td>0.76 ± 0.14</td>
<td>0.003</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>73.8 ± 40.9</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>White blood cells (/mm³)</td>
<td>5100 ± 2300</td>
<td>5100 ± 1800</td>
<td>NS</td>
</tr>
<tr>
<td>Platelet (1000/mm³)</td>
<td>212 ± 72</td>
<td>208 ± 62</td>
<td>NS</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>10 ± 2</td>
<td>12.6 ± 1.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SLEDAI</td>
<td>16 ± 3.8</td>
<td>2.5 ± 1.8</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Abbreviations: SD = standard deviation; DPL = daily protein loss; SLEDAI = SLE Disease Activity Index; ND = not determined; NS = not significant.
Discussion

The present study provides conclusive evidence for an association between serum AECA and nephritis in SLE patients in Taiwan. These findings are consistent with previous studies on SLE patients in Taiwan and in groups from other countries, including the UK, Korea and France [5-9]. In previous studies of SLE patients in Taiwan, Liu and Lei found that patients with cutaneous vasculitis, Raynaud’s phenomenon, or lupus nephropathy tended to have higher mean values of AECA than patients without such manifestations [6]. However, such correlation was not statistically significant in their study. In another report on AECA titer in SLE patients, Li et al identified a 66-kDa membrane antigen recognized by IgG-AECA from patients with lupus nephritis, vasculitis and hypocomplementemia [7]. Although data were not shown, they also mentioned that the highest AECA levels were observed in SLE patients with diffuse proliferative glomerulonephritis, and in patients with proteinuria and nephrotic syndrome, which is consistent with results of the present study and other reports [7]. In addition to predicting the involvement of nephritis and vasculitis, recent evidence indicates that AECA levels are correlated with psychiatric manifestations in SLE patients [10]. Therefore, AECA plays a pivotal role in the pathogenesis of systemic complications of SLE.

Instead of relying only on cell-ELISA, the present study employed both cell-ELISA and cell viability assays to delineate the role of endothelial dysfunction in patients with lupus nephritis. Overwhelmingly, differential AECA titer and anti-endothelial activity in SLE patients with or without nephritis support a role for AECA and anti-endothelial activity as diagnostic markers to detect nephritic states of SLE patients (Fig. 1 and Fig. 2). Moreover, AECA titers and anti-endothelial activities are not only higher in patients with lupus nephritis (Fig. 1 and Fig. 2), but also strongly associated with the SLEDAI (Table 2). Together, these findings strongly support the involvement of endothelial dysfunction during disease progression of SLE.

The pathogenic mechanism of AECA in SLE has been under active investigation. AECA may exert their pathological effects by direct induction of apoptosis in endothelial cells directly by targeting heat-shock protein 60 (Hsp60) [11,12], activation of endothelial cells with increased expression of adhesion molecules [13], and increased secretion of proinflammatory cytokines [13, 14], thereby facilitating the recruitment and trafficking of leucocytes into the inflamed vessels. The role of AECA in promoting inflammation is further strengthened by the recent observation that AECA recognize and bind apoptotic endothelial cells, thereby enhancing the phagocytosis and release of proinflammatory cytokines by macrophages [15].

Table 2. Correlation of anti-endothelial cell antibodies (AECA) and anti-endothelial activities with clinical parameters of systemic lupus erythematosus (SLE) patients

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>AECA</th>
<th>Anti-endothelial proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement 3 (mg/dL)</td>
<td>p&lt;0.001</td>
<td>p&lt;0.003</td>
</tr>
<tr>
<td>Complement 4 (mg/dL)</td>
<td>p&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Anti-double-stranded DNA (IU/mL)</td>
<td>p&lt;0.001</td>
<td>NS</td>
</tr>
<tr>
<td>White blood cells (/mm³)</td>
<td>p&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Platelet (1000/mm³)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>p&lt;0.01</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>SLEDAI</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

Abbreviations: SLEDAI = SLE Disease Activity Index; NS = not significant
In SLE patients with active nephritis, serum AECA titers are not correlated with potency in inhibiting endothelial viability, unlike the case in stable SLE patients or normal controls. This could be attributed to the dramatic changes of circulating cytokines/chemokines levels during nephritic disease, which might stimulate the proliferation or other functions of endothelial cells. Recent studies indicate that serum concentration of angiogenic factors such as hepatocyte growth factor [16] and vascular endothelial growth factor (VEGF) [16-19] were significantly increased in active SLE patients. Besides, a positive correlation between VEGF serum concentration and SLE activity was observed in SLE patients [19]. Finally, serum levels of soluble VEGF receptor-1 are increased, while levels of soluble VEGF receptor-2 are reduced in patients with active SLE [17]. Together with the AECA overproduction, the imbalance between pro- and anti-angiogenic factors may contribute to the vascular deficits in SLE patients.

In conclusion, the present study demonstrates that elevated AECA titer is correlated with SLEDAI disease activity in SLE patients with lupus nephritis. Such correlation sheds light on endothelial dysfunction in the pathogenesis of lupus nephritis. Serum AECA titer may serve as a diagnostic or prognostic marker for SLE. By serial determination of AECA or anti-endothelial activities, future longitudinal prospective studies are warranted to evaluate the potential of these variables for use in monitoring disease activity in SLE patients.

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References

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Fig. 3. Correlation between anti-endothelial cell antibodies (AECA) titer and anti-endothelial activity in: (A) systemic lupus erythematosus (SLE) patients with active nephritis (n =32; p>0.05, r = –0.02); (B) stable SLE patients (n = 32; p=0.074, r = 0.321); and (C) age-matched healthy controls (n = 32; p=0.021, r = 0.42).