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ORIGINAL ARTICLE

Hideaki Bando · Naoto Tamura · Shigeto Kobayashi
Mutsuko Ohyanagi Hara · Yuichi Ichimura
Michiko Tajima · Kazuhiko Haruta · Hiroshi Hashimoto

Endothelial cell-binding antibodies in patients with systemic lupus erythematosus

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Abstract The implications of endothelial cell-binding IgG antibodies (EC IgG) in systemic lupus erythematosus (SLE) was evaluated by determining level of EC IgG in sera from 112 SLE patients. The serum EC IgG level was determined by the cyto-ELISA method using human umbilical vein endothelial cells (HUVEC), human microvascular endothelial cells (HMVEC), and aortic endothelial cells (HAEC) as antigens. The levels of EC IgG were significantly higher among patients with SLE than among healthy control subjects ($P < 0.001$), and 68% (76/112) of SLE patients were shown to be EC IgG-positive. In patients with active lupus nephritis, the level of EC IgG was statistically and significantly elevated compared with those without lupus nephritis ($P < 0.05$). Negative correlations between EC IgG level and levels of CH50, C3, and lymphocyte count were revealed ($P < 0.05$, $P < 0.005$, and $P < 0.05$, respectively). When clinical course was evaluated, the levels of EC IgG correlated with disease activity. Definitive correlations in antibody levels between HUVEC and HMVEC, and between HUVEC and HAEC were revealed (both $P < 0.0001$). The results of this study revealed that the EC IgG in patients with SLE was involved in the onset of clinical manifestation, especially in patients with active lupus nephritis.

Key words Antiendothelial antibodies · Endothelial cell-binding antibodies · Lupus nephritis · Systemic lupus erythematosus (SLE)

H. Bando (✉) · N. Tamura · S. Kobayashi · M. Tajima · K. Haruta · H. Hashimoto
Department of Rheumatology and Internal Medicine, Juntendo University, School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
Tel. +81-3-5802-1067; Fax +81-3-5800-4893
e-mail: bandom@nn.iij4u.or.jp

M. Ohyanagi Hara
Division of Biochemistry, Central Laboratory for Medical Science, Juntendo University, School of Medicine, Tokyo, Japan

Y. Ichimura
Department of Molecular Biology, Toho University, School of Science, Narashino, Japan

Introduction

Systemic lupus erythematosus (SLE) is a disease of unknown etiology in which tissues and cells are damaged by pathogenic autoantibodies and immune complexes. SLE is associated with a variety of vascular involvements and its pathogenesis is widely diverse.[1] Antiendothelial cell antibodies (AECA) or endothelial cell-binding IgG antibodies (EC IgG) have been determined in various types of autoimmune diseases associated with vasculitis.[2–4] AECA or EC IgG have been detected in more than 50% of patients with SLE, and its correlation with disease activity has been reported.[5–9] However, the results of the relationship with clinical parameters such as levels of serum complement, antinuclear antibody (ANA), anti-DNA antibody (anti-DNA Ab), and antiphospholipid antibody (APA) differed in many reports.[5,6,10–12] For the determination of EC IgG, the cyto-ELISA method is routinely utilized. In most experiments, human umbilical vein endothelial cells (HUVEC) were used as the antigen. There has been no report describing its reactivity to other types of endothelial cells (EC).

We determined the level of IgG EC IgG in sera of SLE patients by the cyto-ELISA method to investigate its relation to laboratory parameters and SLE disease activity index (SLEDAI).[13] Furthermore, the study was performed on the reactivity to EC obtained from large vessels and vessels of microvascular origin.

Patients and methods

Patients

One hundred and twelve SLE patients treated in the outpatient clinic or the ward of the Department of Rheumatology, Juntendo University Hospital, from 1995 to 1998 were evaluated. All patients fulfilled the criteria for SLE proposed by the American College of Rheumatology (formerly the American Rheumatism Association).[14] The age of the patients ranged from 18 to 52 years, with mean of 35.2 years.

The male-to-female ratio was 1 : 7, and the mean duration of disease was 3.6 years. The activity of lupus nephritis was scored with the SLEDAI, and judging from clinical and laboratory examinations.[15] Sera from 24 healthy control subjects, with ages ranging from 23 to 40 years (mean 33.0 years), were also determined.

Methods

Cell culture

Human umbilical vein endothelial cells (HUVEC; Sanko Pure Chemical Industry, Tokyo, Japan) were cultured on type II collagen-coated dishes. Medium 199 (ICN Biomedicals, Aurora, OH, USA) with heparin (5000 U/l), $NaHCO_3$ (2.6×10^{-2} M/l), Hepes (5.0×10^{-3} M/l), penicillin-streptomycin (Gibcobral, Grand Island, NY, USA), endothelial cell growth supplement (Upstate Biotechnology, Lake Placid, NY, USA) (1.9×10^{-2} mg/l), and 10% fetal bovine serum (FBS) was used for the culture. HUVEC was used after the second subculture. Microvascular endothelial cells (HMVEC) and aortic endothelial cells (HAEC) were purchased (Sanko Pure Chemical Industry, Tokyo, Japan). Synovial cells of patients with rheumatoid arthritis (RA), collected from synovium which was resected during surgery, were used for culture.[16]

Determination f EC IgG level

Endothelial cells (EC) (HUVEC, HAEC, HMVEC) were cultured on a collagen-coated 96-well microplate (Celltite C-1 Plate 96F; Sumitomo Bakelite, Akita, Japan) until the cells reached the confluent state, and then fixed with 0.1% glutaraldehyde in phosphate-buffered saline (PBS). After washing with 0.05% Tween 20–PBS, the wells were incubated with Block Ace (Snow Brand, Tokyo, Japan) for at least 1 h at room temperature (RT). After washing with 0.05% Tween 20–PBS, IgG (100 μg/ml), which was purified with serum containing protein G sepharose, was allowed to react at RT for 1 h. Determination was performed using biotinized goat antihuman IgG (γ) antibody (Vector Laboratories, Burlingame, CA, USA) and then, after washing the wells, peroxidase-labeled avidin (Zymed Laboratories, South San Francisco, CA, USA) was added. The colorimetric reaction was developed with *o*-phenylenediamine. Absorbance at a wavelength of 490 nm/620 nm optical density (OD) was measured with a micro-ELISA reader. The EC IgG level was defined as: (The average value of identical specimens from two wells) − (The average value of one negative control from two wells). Monoclonal antiendothelial cell antibodies (10B9, American Tissue Culture Collection, Manassas, VA, USA) were used as a positive control, and interplate differences were compensated for.

For the absorption analysis, the sera of EC IgG-positive patients at 1 : 100 dilution were added to HUVEC, synovial cells, and lymphocytes from healthy control subjects (5×10^6 cells each), and reacted for 2 h at 4°C. The supernatant of the mixture was collected and the absorption procedure was repeated twice. The titer of EC IgG in the supernatant was determined with cyto-ELISA. The absorption ratio was expressed as: (EC IgG level before absorption) − (EC IgG level after absorption)/(EC IgG level before absorption) (%).

Statistical analysis

The Mann–Whitney test was used for comparisons between the two groups. Concerning EC IgG levels and clinical parameters, correlation analysis was performed with Spearman's rank correlation coefficient.

Results

Serum EC IgG levels in SLE patients

Serum EC IgG levels were determined in 112 SLE patients and compared with those of 24 healthy control subjects. The mean optical density (OD) of EC IgG (mean ± SD, 0.401 ± 0.133) in SLE patients was significantly higher than that of healthy control subjects (0.276 ± 0.045, $P < 0.001$, Fig. 1). When the OD of the mean +2SD of healthy control subjects was defined as the cut-off value, 68% (76/112) of SLE patients were shown to be EC IgG-positive.

Specificity of the method

To confirm the specificity of the cyto-ELISA method, sera of ten EC IgG-positive patients were preincubated with

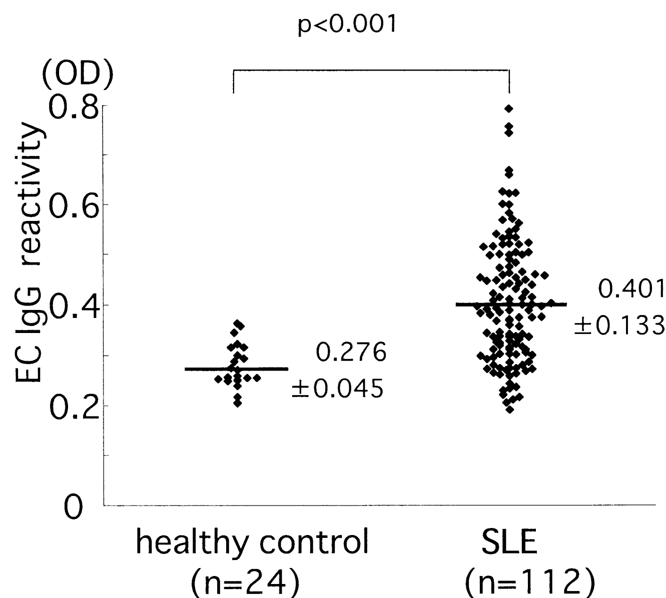

Fig. 1. EC IgG level in patients with systemic lupus erythematosus (*SLE*). EC IgG level was determined in sera of SLE patients and healthy control subjects by the cyto-ELISA method using human umbilical vein endothelial cells (HUVEC) as the antigen. The mean value, allowing for absorbance at optical density (*OD*) (490 nm/620 nm), was defined as the antibody level. In 112 SLE patients, the mean ± SD of their EC IgG levels was 0.401 ± 0.133, which was significantly higher than the 0.276 ± 0.045 of 24 healthy control subjects ($P < 0.001$). The bar indicates the mean value, and the numerical value against it is the mean ± SD

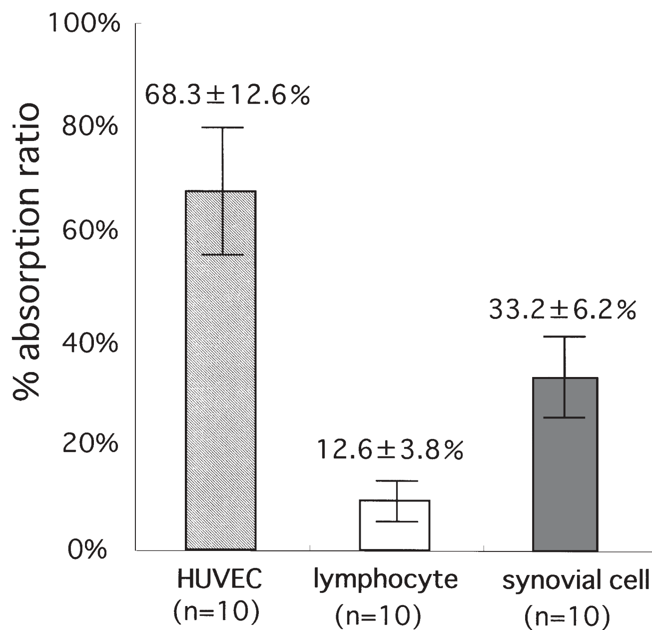

Fig. 2. Absorption experiment on EC IgG levels. EC IgG-positive sera at 1:200 dilution from ten SLE patients were preincubated with *HUVEC*, lymphocytes, or synovial cells (5×10^6 cells). Then the supernatant was analyzed by the cyto-ELISA method. The absorption ratio was defined as: (EC IgG titer before absorption) − (EC IgG titer after absorption)/(EC IgG titer before absorption) (%), and the absorption ratio was compared according to cell type. The absorption ratios (mean ± SD) were 78.3 ± 12.6%, 12.6 ± 3.8%, and 33.2 ± 6.2%, respectively. The numerical value on the bar is the mean ± SD

HUVEC, peripheral lymphocytes, or synovial cells, and then EC IgG levels were determined. The reduction of EC IgG levels from the mean value of 68.3% ± 12.6% was shown when the sera were absorbed by HUVEC, although only 12.6% ± 3.8% were reduced by peripheral lymphocytes. The EC IgG level was partially absorbed by synovial cells (Fig. 2).

Next, IgG which was purified from sera of eight SLE patients was checked for EC IgG levels. The EC IgG level from IgG of eight SLE patients was closely correlated with that of the serum from each patient ($r = 0.953$, $P < 0.001$; data not shown).

Relationship between the level of EC IgG and disease activity

The SLE disease activity index (SLEDAI) was determined in 58 patients to assess the relationship between disease activity and EC IgG levels. As shown in Fig. 3, EC IgG levels were statistically correlated with SLEDAI ($P < 0.05$) although the correlation coefficient was shown to be quite small ($r = 0.279$). Among the 58 patients, 20 cases had active lupus nephritis. When EC IgG levels were compared between patients with and without active lupus nephritis, the level was statistically and significantly higher among the cases with active lupus nephritis (0.437 ± 0.074) than among those without lupus nephritis (0.376 ± 0.089; $P < 0.05$) (Fig. 4).

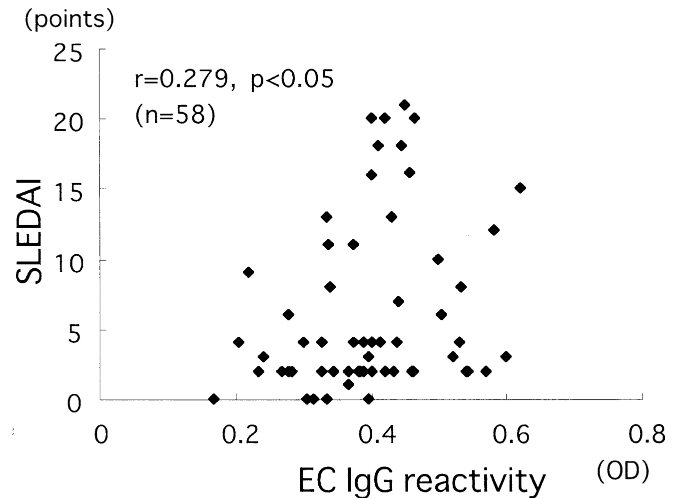

Fig. 3. Correlation between EC IgG levels and SLE disease activity index (*SLEDAI*). A correlation was found between each SLEDAI value and the EC IgG level in patients with SLE ($n = 58$) ($P < 0.05$)

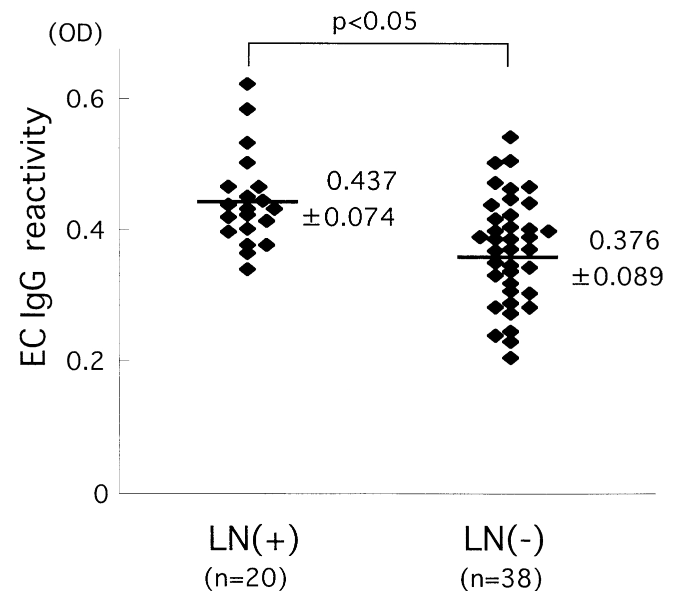

Fig. 4. Comparison of EC IgG levels depending on the presence or absence of active lupus nephritis (*LN*). EC IgG level was compared between patients with and without active lupus nephritis. For patients with active lupus nephritis (LN+; $n = 20$), the EC IgG level was 0.437 ± 0.074 (mean ± SD). This was significantly higher than the 0.376 ± 0.089 for patients without lupus nephritis (LN−; $n = 38$) ($P < 0.05$). The bar indicates the mean value, and the numerical value is the mean ± SD

Relationship with laboratory findings

The relationship between EC IgG level and laboratory findings was assessed. A significant negative correlation between the level of EC IgG and levels of CH50 and C3 was revealed ($P < 0.05$ and $P < 0.005$, respectively; Fig. 5). In addition, a negative correlation with lymphocyte count was shown ($P < 0.05$; data not shown). However, there was no statistical correlation between the level of EC IgG and the

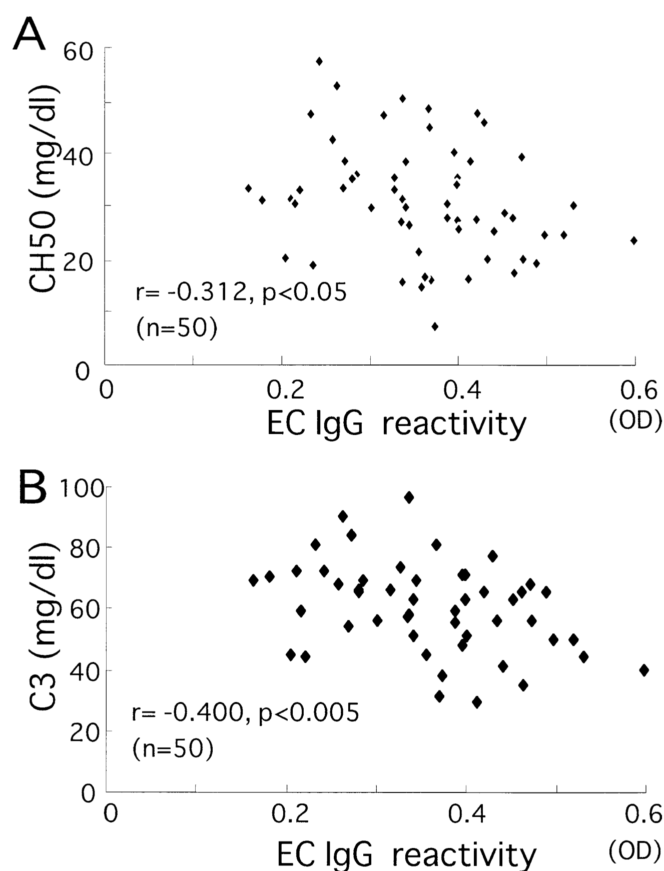

Fig. 5. Correlation of EC IgG level with CH50 and C3. The correlation of EC IgG level with serum CH50 (**A**) and C3 (**B**) was evaluated in patients with SLE. A significant negative correlation was found in both cases (CH50, $P < 0.05$; C3, $P < 0.05$)

levels of anti-DNA Ab and anti-β2GPI-dependent anticardiolipin antibody (aCL). Furthermore, no differences were noted in the level of anti-DNA Ab between EC IgG-positive patients and EC IgG-negative patients, or in the level of EC IgG between lupus anticoagulant (LA)-positive and -negative patients (data not shown).

Longitudinal study

In 12 patients, EC IgG levels were determined more than twice during their clinical course. In 8 of 12 patients, the level of EC IgG changed in relation to the disease activity (Fig. 6). EC IgG levels at exacerbation increased significantly compared with levels prior to exacerbation, or decreased significantly after treatment ($P < 0.05$ and $P < 0.01$, respectively). Six of eight patients had exacerbation of lupus nephritis. Of the four patients who did not show any change in SLEDAI, no conspicuous changes in EC IgG levels were demonstrated (data not shown).

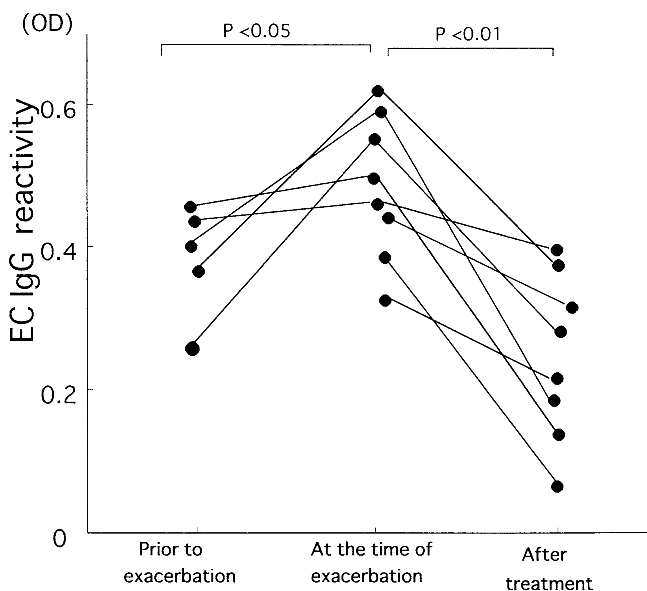

Fig. 6. Changes in EC IgG level in relation to disease course. The differences in EC IgG level were evaluated in eight cases which showed changes in disease activity during the period of observation. Exacerbation was defined as an increase in SLEDAI of 10 points or more, and improvement was defined as a decrease in SLEDAI of 5 points or more after treatment. The EC IgG level of five patients increased from 0.378 ± 0.075 to 0.548 ± 0.084 ($P < 0.05$) at the time of exacerbation, and the EC IgG level of eight patients decreased from 0.490 ± 0.105 to 0.268 ± 0.126 ($P < 0.01$) after treatment. Of the exacerbation cases, six were of active lupus nephritis. In all cases, the changes in SLEDAI and EC IgG level coincided

Reactivity of EC IgG to microvascular endothelial cells and aortic endothelial cells

Microvascular endothelial cells (HMVEC) and aortic endothelial cells (HAEC) were then used as the antigen to study the difference in reactivity other than that of HUVEC. Serum reactivity was determined in 20 patients with SLE by the same procedure (cyto-ELISA). Regardless of which cells (HMVEC or HAEC) were used, the EC IgG levels determined coincided with the results obtained using HUVEC. A definitive correlation was shown between the antibody levels of HUVEC and HMVEC, and of HUVEC and HAEC (both $P < 0.0001$) (Fig. 7).

Discussion

The determination of EC IgG or AECA has been reported not only in SLE patients, but also in patients with autoimmune diseases with vascular lesions, such as systemic scleroderma, Kawasaki disease, antiphospholipid antibody syndrome, etc., and the clinical and pathological implications of EC IgG have been reported.[2,4] The term "EC IgG" instead of AECA has been used since Chan and Cheng[3] demonstrated that anti-DNA antibodies bind to EC indirectly through immunoglobulin-bounded DNA or DNA-binding proteins on the EC membrane. Furthermore, some reports have shown that AECAs also react to peripheral

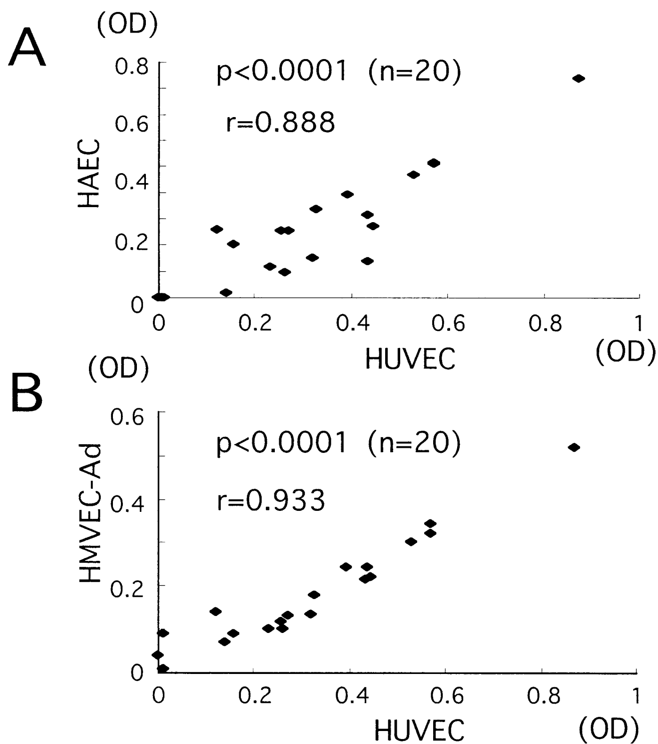

Fig. 7. Evaluation of cellular specificity of EC IgG level. EC IgG level was determined in 20 SLE patients by the cyto-ELISA method using (**B**) human microvascular endothelial cells (*HMVEC*) and (**A**) human aortic endothelial cells (*HAEC*) as antigens, and the relation of antibody level with *HUVEC* was assessed. These relations showed a definitive correlation of the antibody level with HUVEC as the antigen (both $P < 0.0001$)

blood mononuclear cells, synovial cells, and fibroblasts, even though the nonspecific binding of AECA via Fc receptor has been ruled out.[2] Although EC IgG was not strictly distinguished from AECA by some investigators,[2–4] we prefer the use of EC IgG since our results did not prove that out assay determined EC-specific antibodies.

In the cyto-ELISA method, HUVEC was fixed with 0.1% glutaraldehyde, although there was no difference in the positive ratio of EC IgG in SLE patients regardless of whether HUVEC was fixed or not, as revealed in a preliminary study. The OD value tended to be increased in sera of healthy control subjects with the fixing procedure, but there was an apparent difference in OD values between the sera from SLE patients and from healthy control subjects. If HUVEC was not fixed, there was a tendency for the cells to become detached during the washing procedure of ELISA, and this led to an impairment of the accuracy of the results. When fixed cells were used, a reaction to the nuclear components or protein components inside the EC is postulated to have occurred. However, the results revealed that nonspecific reaction to these molecules exerts no influence because EC IgG was absorbed by unfixed HUVEC (see Fig. 2), and also because no relation with ANA or anti-DNA Ab levels was identified (data not shown). A reduction of EC IgG levels in the mean value of 33.2% was demonstrated when the EC IgG was absorbed by synovial cells. Since EC IgG binds to heterogeneous EC antigens, it has been reported that EC IgG is partially absorbed by peripheral blood mononuclear cells and fibroblasts.[2]

We studied the serum EC IgG level in 112 SLE patients by the cyto-ELISA method using HUVEC. The level of EC IgG among SLE patients was significantly higher than that of healthy control subjects (see Fig. 1). Seventy-six (68%) of 112 patients were positive for EC IgG. These results coincided with those of previous reports from other countries.[17–19] This study showed a statistical correlation between EC IgG level and SLEDAI (see Fig. 3). Furthermore, the EC IgG level had a negative correlation with the levels of CH50, C3, and lymphocyte counts. In addition, in the longitudinal study, there was a tendency for EC IgG levels to change in relation to SLEDAI (see Fig. 6). However, the correlation coefficient was shown to be small between EC IgG and SLEDAI ($r = 0.279$), CH50 ($r = -0.312$), and C3 ($r = -0.400$).

The level of IgG-AECA or EC IgG in SLE patients is reported to be closely related to the manifestation of pulmonary hypertension, Raynaud's phenomenon, digital ulcer, and nephritis.[4,12,20,21] In this study, a significant difference in the EC IgG levels was found between patients with active lupus nephritis and those without nephritis (see Fig. 4). Therefore, the implication of EC IgG in relation to SLEDAI is believed to be defined by certain clinical manifestations in SLE, such as lupus nephritis or pulmonary hypertension.[5–8]

The difference in the reactivity of EC IgG to the type of EC used was assessed using EC derived from large vessels and from microvascular vessels. These cells may differ from HUVEC in the type or amount of molecules expressed on the surface of the cells, but there was no difference in EC IgG levels determined by the cyto-ELISA method in reference to cell type (see Fig. 7).

As corresponding antigens to EC IgG, a number of candidates, such as heparin sulfate, thrombomodulin, CD36, etc., have been reported.[22] EC IgG is not an autoantibody which is specific to a single antigen, and therefore the level of EC IgG is considered to be the total reaction of antibodies to numerous cell surface molecules on the EC. Since it has been reported that a variety of autoantibodies are produced by SLE patients, some of these autoantibodies may be cross-reactive or polyreactive with molecules on the EC, especially negatively charged molecules.[23]

Several investigators have reported the pathogenesis of EC IgG or AECA in SLE. AECA is known to have complement-dependent cytotoxicity,[2] and up-regulates the expression of E-selectin, ICAM-1, and VCAM-1 on EC.[24,25] It has also been reported that AECA derived from sera of SLE patients reinforces the expression of adhesion molecules on monocytes, and further enhances the production and secretion of inflammatory cytokines.[24–27] Monoclonal AECA derived from mouse reacts to EC and induces the production of antiphospholipid antibodies through an idiotype–antiidiotype network.[28] Furthermore, AECA in SLE is reported to induce the production of endothelin-l.[29] Therefore, AECA induces a proinflammatory and procoagulant state through the activation of EC as well as being cytotoxic to EC.

Based on these reports, EC IgG or AECA is postulated to have an important role in vascular injury among SLE patients. Further analysis of EC IgG, and especially its clinical and pathogenical implications in SLE, are necessary, and are currently underway in this laboratory.

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