ABSTRACT

Objectives To investigate the prevalence of anti-endothelial cell antibodies (AECA) and antiphospholipid antibodies, and the correlations of their isotype distributions and titers with disease activity in patients with Takayasu’s arteritis (TA).

Methods. Forty-seven patients with TA and 30 age- and sex-matched controls were studied. Blood samples were obtained from all patients and they were divided into either active or stable disease groups. Paired samples were available in 18 patients at both active and stable stage, respectively. AECA against human umbilical vein endothelial cells and antiphospholipid antibodies were measured.

Results. Forty-two (89.4%) TA patients had AECA, and positivity rates of IgM and IgG AECA were 83.0% and 68.1%, respectively, while those for controls were both 3.3%. The titers of IgM and IgG AECA in patients were significantly higher than those in controls. IgM AECA titers of the active group were significantly higher than those of the stable group, but IgG AECA titers were not.

Conclusion IgM AECA and IgG AECA were more prevalent and their titers were higher in patients with TA than in controls, and IgM AECA titers correlated well with the disease activity of TA. Antiphospholipid antibodies were not found significant.

Introduction Takayasu’s arteritis (TA) is a chronic inflammatory disease of the large elastic arteries that primarily affects the aorta and its main branches, resulting in luminal stenosis and aneurysmal changes in the large vessels (1, 2). The etiology of TA is still unknown, but previous studies suggested that an autoimmune mechanism plays an important role in the pathogenesis of the disease (3, 4). The facts that TA has been linked to other autoimmune diseases, such as systemic lupus erythematosus (SLE) and adult onset Still’s disease (5, 6), and that the disease shows a favorable response to immunosuppressive treatment (7, 8) support the autoimmune mechanism in the pathogenesis of TA. However, despite many studies aimed to evaluate the immunological background in the disease, the autoimmune mechanism in the pathogenesis of TA has not been elucidated clearly.

As a heterogeneous group of antibodies directed against a variety of antigen determinants on the vascular endothelial cells, anti-endothelial cell antibodies (AECA) have been detected in the diseases showing diverse immunemediated vascular damage. There is increasing evidence to show that AECA might be pathogenic in autoimmune vascular diseases, such as Wegener’s granulomatosis, microscopic polyangiitis, Kawasaki disease, Behçet’s disease, and SLE (9, 10), and it is relevant to note that the presence and titer of AECA has been correlated with disease activity in some of these diseases (12, 13, 14). Previous studies showed AECA are able to up-regulate the expression of adhesion molecules and to induce the secretion of cytokine and chemokine which, in turn, cause leukocyte recruitment and adhesion, as well as being cytotoxic to endothelial cells (18-20). A previous idiotypic animal model has provided further evidence that AECA can be pathogenic (21). With this regard, several studies evaluated the...
role of AECA in TA, and showed that 33-94% of patients with TA have AECA and AECA titer can correlate well with the disease activity of TA, suggesting possible pathogenic role of AECA in the disease (17, 22-25).

Antiphospholipid antibodies are associated with recurrent vascular thrombosis and can be found in the sera of patients with certain vasculitic disorders, including giant cell arteritis, Wegener’s granulomatosis, and polyarteritis nodosa (26, 27). Also, it was reported that antiphospholipid antibodies can induce endothelial cell activation and vascular damage (28, 29). But reports regarding their role in the pathogenesis of TA are conflicting. In previous studies, antiphospholipid antibodies were reported to be associated with TA (22, 30), but this observation was not repeated in another study that aimed to confirm the pathogenic role of antiphospholipid antibodies in TA (31).

Vascular inflammation and intimal proliferation leading to the vascular stenosis or occlusion are the main characteristics of TA, and intraluminal thrombosis can be also encountered in TA (2). However, the role of AECA and antiphospholipid antibodies in pathophysiology of the disease has not been determined precisely. The studies on serial changes of AECA involving an adequate number of patients are lacking, and the reports on antiphospholipid antibodies showed an inconsistency regarding their presence and roles in patients with TA. In this study, we evaluated the prevalence of these antibodies in patients with TA and investigated if their isotype distributions and titers correlate with the disease activity of TA.

Patients and methods

Patients

Forty-seven patients (3 men and 44 women, mean age: 33.9 ± 12.7 years), who were newly diagnosed with TA according to the American College of Rheumatology 1990 criteria for the classification of TA (32), were enrolled in this study. All the patients were seen at Yonsei University Medical Center, Seoul, Korea from January 2000 to January 2003. Thirty age- and sex-matched healthy persons without any evidence of connective tissue disease (2 men and 28 women, mean age: 34.4 ± 10.3 years) were enrolled as the controls. Blood samples were collected from all patients at study enrollment and they were divided into either active or stable disease groups. From 18 patients who had active disease initially and became remitted after treatment, paired blood samples were collected at initial active stage and at stable stage after treatment. All assessments of disease activity were performed according to the National Institute for Health (NIH) criteria for active disease (1). These criteria include: constitutional symptoms, such as fever, musculoskeletal symptoms; elevated erythrocyte sedimentation rate (ESR, Westergren method); features of vascular ischemia or inflammation, such as claudication, diminished or absent pulse, bruit, vascular pain, and blood pressure difference in either upper or lower extremities; and typical angiographic findings. New onset or worsening of two or more features defined ‘active disease’ and decrease of symptoms and signs or complete resolution of clinical features was indicative of stable disease. Remission was defined as having stable disease during the course of disease in those who previously had active disease, and relapse was defined as having active disease during the course of disease in those who had experienced stable disease. Remission and relapse lasting more than 3 months were eligible for analysis. Demographic data, including sex, age and disease duration, were also evaluated. The assessments of clinical activity and demographic data were performed at the time of sample collection for the measurements of biochemical variables. This study was approved by our ethics committee, and all study participants, as well as all donors of umbilical cord, provided their informed consent.

Isolation and culture of cells

The human umbilical vein endothelial cells (HUVEC) were isolated from the normal term human umbilical cord veins. Human umbilical cords were obtained from the Department of Gynecology and Obstetrics of our institute. Fresh umbilical cords were placed in Hank’s balanced salt solution (HBSS) and kept in ice for transport. One end of cord was cannulated with an angiocatheter and secured with a plastic umbilical cord tie. Umbilical vein was washed thoroughly with HBSS until no more red blood cells were seen in the eluate. Then, 50 ml of freshly prepared collagenase 0.1% in HBSS was installed for 10 minutes. After the perfusion of collagenase, the cord was incubated in 100 ml of HBSS for 15 minutes and washed 3 times in 100 ml of HBSS to remove any adherent cells on surface. Collagenase and loosened endothelial cells were rinsed out by installing 50 ml of HBSS through catheter and drained into conical tube. Then, 10 ml of inactivation media was added to the conical tube and cell pellet was spun down at 1,200 rpm for 10 minutes. After removing the supernatant, 50 ml of inactivation media was added and the pellet was resuspended in endothelial basal media (Clonetics, San Diego, CA, USA), with 5 ng/ml of epidermal growth factor (Clonetics), 1 µg/ml of hydrocortisone acetate (Sigma, St. Louis, MO, USA), 5 x 10^-5 M dibutyryl cAMP (Sigma), 100 units/ml of penicillin, 100 µg/ml of streptomycin, 250 µg/ml of amphotericin B (Sigma), and 30% human serum (Irvine Scientific, Santa Ana, CA, USA). The resultant cell cultures were free of contaminating fibroblasts, as assessed by immunofluorescent staining with rabbit anti-human factor VIII related antigen antibody (Behring, Marberg, Germany). Until required for experiments, cells from the second passage were cultured in endothelial basal medium (Clonetics) containing 5 µg of human epidermal growth factor, 1 µg/ml of hydrocortisone acetate, 100 µg/ml of gentamicin, 250 µg/ml of amphotericin B, 5 x 10^-5 M dibutyryl cAMP, 100 units/ml of penicillin, and 2% fetal bovine serum.

Measurement of AECA

HUVECs were plated in microtiter plates and allowed to grow to confluence over 24 hours. The plates were washed 3 times with phosphate buffered saline (PBS, Sigma), pH 7.4 and cells were fixed with glutaraldehyde for
20 minutes at 20°C. After washing for three times with PBS, 100 µl of sera from normal controls and patients with TA, diluted 1:50 in HBSS with divalent cations (Irvine Scientific) and 1% bovine serum albumin (BSA; Sigma), was added to each well and the plates were incubated for 1 hour at 37°C. After three washes, 100 ml of peroxidase-conjugated goat anti-human IgG or IgM diluted 1:1,000 in HBSS with divalent cations and 1% BSA was added to each well, and the plates were incubated for 1 hour at 37°C. Three wells containing diluent only were used as blanks to provide a background level, and three positive and three negative control sera were included on each plate. Antibody binding was quantified colorimetrically by adding tetramethylbenzidine (Sigma) as substrate. One microliter of 30% H2O2 was added immediately prior to use. The chromogenic reaction was stopped with 8N H2SO4, and the plates were read spectrophotometrically at 450 nm on an ELISA reader (Dynatech, Alexandria, VA). Positivity was defined as an optical density (OD) greater than the mean plus 2 standard deviations for the control sera.

Measurement of antiphospholipid antibodies
A semi-quantitative indirect enzyme immunoassay was used to measure IgG and IgM anticardiolipin antibodies (Zeus Scientific, Inc., Raritan, NJ, USA), and IgM and IgG anti-β2-glycoprotein I antibodies (Neogen corp., Lansing, MI, USA) in patients with TA and controls using commercial ELISA kits. The positivity of the lupus anticoagulant was determined according to the guidelines of the International Society on Thrombosis and Hemostasis Scientific Subcommittee on lupus anticoagulants/phospholipid-dependent antibodies (33) using a commercial lupus anticoagulant kit (Instrumentation Laboratory Company, Lexington, MA, USA).

Other laboratory tests
The white blood cell (WBC) count, erythrocyte sedimentation rate (ESR, modified Westergren method) and C-reactive protein (CRP) levels were measured in all patients with TA and the controls. In 18 patients whose paired samples were available, the WBC count, ESR, and CRP were measured at the same time point with the collection of their samples for the measurement of AECA and antiphospholipid antibodies.

Statistical analysis
The results (mean ± standard deviation) represent duplicate measurement. The comparisons of demographic data and laboratory data between active disease group and stable disease group at enrollment were performed using independent t-test or chi-square test. An ANOVA test with multiple comparison method was used to determine the differences in the prevalence and the titers of the antibodies among the active disease group, the stable disease group and the controls. The Pearson’s correlation test was used to analyze the correlation between the AECA titers and the other laboratory parameters. In patients whose paired samples were collected, the Wilcoxon’s rank test was used to determine the differences in the prevalence and titers of the antibodies between the active stage and stable stage, and the Spearman’s rank correlation test was performed to determine the correlations among the changes in the AECA titers and the changes in the other laboratory parameters. A p value < 0.05 was considered statistically significant.

Results
Patient characteristics
The patients with TA were divided into two groups according to the disease activity; active disease group (n = 26) and stable disease group (n = 21). Sex distributions, mean age, and mean disease duration were not different between active and stable disease group. Among laboratory parameters, ESR were more frequently elevated in active disease group and their titers were significantly higher than those in stable disease group, however, positivities and titers of CRP and leukocytosis were not different between two groups (Table 1). In 18 patients whose paired samples were available, glucocorticoids were administered to all patients and immunosuppressive agents, such as methotrexate or azathioprine, were added to glucocorticoids in 10 of them. During mean follow-up duration of 10.7 ± 6.7 months, remissions were achieved in all of them.

Table 1. Characteristics of TA patients and outcomes of patients with paired blood samples.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Active group (n = 26)</th>
<th>Stable group (n = 21)</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (men/women)</td>
<td>2 / 24</td>
<td>1 / 20</td>
<td>NS</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>33.4 ± 13.0</td>
<td>34.1 ± 12.0</td>
<td>NS</td>
</tr>
<tr>
<td>Mean disease duration (months)</td>
<td>40.3 ± 35.7</td>
<td>41.0 ± 35.0</td>
<td>NS</td>
</tr>
<tr>
<td>Laboratory findings</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elevated ESR, n (%)</td>
<td>23/26</td>
<td>5/23</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Mean ESR level (mm/hour)</td>
<td>41.1 ± 18.8</td>
<td>41.4 ± 9.6</td>
<td>0.01</td>
</tr>
<tr>
<td>Elevated CRP, n (%)</td>
<td>15/26</td>
<td>6/28</td>
<td>NS</td>
</tr>
<tr>
<td>Mean CRP level (mg/dL)</td>
<td>1.2 ± 1.1</td>
<td>0.6 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Elevated WBC count, n (%)</td>
<td>16/26</td>
<td>5/23</td>
<td>NS</td>
</tr>
<tr>
<td>Mean WBC count ( /mm3)</td>
<td>11,300 ± 5,450</td>
<td>8,850 ± 4,810</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Calculated using independent t-test or Chi-square test.

ESR: erythrocyte sedimentation rate (reference value: < 15 mm/hour in men and < 20 mm/hour in women). CRP: C-reactive protein (reference value: < 0.8 mg/dL). WBC: white blood cell count (reference value: < 10,800/mm3). NS: not significant.
and 0.21 ± 0.07, respectively, and those of the controls were 0.13 ± 0.04 and 0.08 ± 0.02, respectively. The mean OD values of both IgM and IgG AECA were significantly higher in patients with TA than in controls (both p < 0.05) (Fig. 1).

In patients with TA, the sex distributions, mean age, mean disease durations, mean ESR, mean CRP level, and mean WBC counts were not different between those with AECA and those without AECA.

### Disease activity and AECA

IgM and IgG AECA were detected in 24 (92.3%) and 18 (69.2%) patients of active disease group, and in 14 (66.7%) and 13 (61.9%) patients of stable disease group. IgM AECA were more prevalent in active disease group than in stable disease group (p < 0.05), but the positivity rate of IgG AECA did not show a significant difference between two groups (Table II). The mean OD value of IgM AECA for active disease group was 0.40 ± 0.12 and that for stable disease group was 0.25 ± 0.10. Both were significantly higher than those of the controls and the mean OD value of IgM AECA for active disease group was significantly higher than that for stable disease group (p < 0.05) (Fig. 2). The mean OD value of IgG AECA for active disease group was 0.24 ± 0.11 and that for stable disease group was 0.20 ± 0.09. Although the mean OD values of IgG AECA in active disease group and in stable disease group were significantly higher than that of the controls, the difference between active and stable disease group was not statistically significant (Fig. 2). The OD values of IgM AECA and IgG AECA did not correlate with the age, disease duration, WBC count, ESR, and CRP levels in patients with TA.

In 18 patients whose paired samples were available, the mean OD values of IgM AECA at active and stable stage were 0.44 ± 0.10 and 0.28 ± 0.10, respectively. The mean OD value of IgM AECA at active stage was significantly higher than that at stable stage (p < 0.05). The mean OD values of IgG AECA at active and stable stage were

Table II. Positivity rates of the antibodies in patients with Takayasu’s arteritis.

<table>
<thead>
<tr>
<th></th>
<th>Active disease group (n = 26)</th>
<th>Stable disease group (n = 21)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>IgM AECA</td>
<td>24</td>
<td>92.3</td>
<td>14</td>
</tr>
<tr>
<td>IgG AECA</td>
<td>18</td>
<td>69.2</td>
<td>13</td>
</tr>
<tr>
<td>IgM anti-β2 GPI</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IgG anti-β2 GPI</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>IgM aCL</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>IgG aCL</td>
<td>1</td>
<td>3.8</td>
<td>1</td>
</tr>
<tr>
<td>LAC</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

AECA: anti-endothelial cell antibodies, anti-β2 GPI: anti-β2 glycoprotein I antibody, aCL: anticardiolipin antibody, LAC: lupus anticoagulant, NS: not significant.

Fig. 1. Comparison of isotype distributions and their titers of AECA between patients with TA and controls. IgM and IgG AECA titers in patients with TA were 0.32 ± 0.12 and 0.21 ± 0.10, respectively, and those in control group were 0.13 ± 0.04 and 0.08 ± 0.02, respectively. The mean OD values of IgM and IgG AECA in patients with TA were significantly higher than those in control group (p < 0.05). Dots represent the mean values and error bars represent two standard deviations.

Fig. 2. Comparison of isotype distributions and their titers of AECA between active and stable disease group. IgM AECA titers were 0.40 ± 0.13 in active disease group and 0.28 ± 0.10 in stable disease group, and IgG AECA titers were 0.24 ± 0.11 in active disease group and 0.20 ± 0.09 in stable disease group. IgM AECA titer was significantly higher in stable disease group than in stable disease group (p < 0.05), but IgG AECA titer did not show significant difference between two groups. Dots represent the mean values and error bars represent two standard deviations. NS: not significant.
Prevalence and titers of antiphospholipid antibodies

IgG anti-β2-glycoprotein I antibody was positive in one patient with TA. In that patient, the activity of TA was assessed to be stable and IgG anti-β2-glycoprotein I antibody titer was low. However, IgM anti-β2-glycoprotein I antibody was not found in any patient with TA. In controls, both IgG and IgM anti-β2-glycoprotein I antibody were not detected.

IgM anticardiolipin antibody was positive in one patient with TA and IgG anticardiolipin antibody was positive in two patients. In a patient in whom IgG anticardiolipin antibody was positive, the disease activity of TA was active, whereas the other two patients had the stable disease. No IgM and IgG anticardiolipin antibody was detected in the controls.

The lupus anticoagulant was not detected in any of the patients with TA and controls (Table II).

Discussion

Most patients with TA present their symptoms and signs associated with stenosis and occlusion of large vessels at the time of diagnosis. However, the surrogates for the early detection of vascular inflammation causing luminal stenosis are lacking and this may be partly attributed by the unknown pathogenesis of the disease. Although previous investigations for antinuclear antibodies, anti-neutrophil cytoplasmic antibodies, and rheumatoid factor have failed to document their roles in the pathogenesis of the disease (34, 35), several studies reported that AECA were present in 33-94% of patients with TA (17, 22-25). In the present study, similarly to the prevalence reported by Sima et al. and Eichhorn et al. (23, 24), 89.4% of our patients with TA were found to have IgM and/or IgG AECA and the titers of both IgM and IgG AECA were significantly higher than those from controls, which suggest the possible pathogenic role of these antibodies in TA.

A previous study showed that the complement mediated cytotoxicity by AECA might have an important role in vascular damage observed in TA (36), and the pathogenic role of AECA in systemic vasculitis is supported by an animal model of autoantibody-induced vasculitis, in which immunofluorescent staining of vascular lesion showed the immunoglobulin deposition at vessel wall (21). AECA bind to surface proteins on cell membrane of endothelial cells and it is known that there are likely to be multiple target antigens (37, 38), and a recent report by Tripathy et al. showed an association between annexin V antibodies and AECA and suggested the presence of annexin V as one of the possible target antigens of AECA in TA (39). Accordingly, sera positive for AECA display a broad reactivity against endothelial cells obtained from different anatomic sources: from large vessels, including HUVEC and saphenous vein, as well as from small vessels such as renal, skin, omental, and brain microvasculature (40), and AECA in TA was found to bind to and activate macrovascular endothelial cells, including HUVECs, but not microvascular endothelial cells (25). It has been also reported that the patients with TA had elevated AECA and their levels correlate with disease activity of the disease (17, 22-25).

However, most of previous series enrolled small numbers of patients and performed in a cross-sectional manner, showing greatly varying prevalence of AECA among studies. To overcome these limitations, we enrolled a relatively large population of patients and paired blood samples were used to identify the correlations of AECA with disease activity. Thus, in the present study, we found that the prevalence and the titers of IgM AECA in active disease group were higher than in stable disease group, and the titers of AECA that were measured using the paired serum samples showed a significant decrease in the IgM AECA titer at stable stage after treatment and the changes in IgM AECA titers correlated well with those of ESR levels, suggesting that AECA may contribute to the pathogenesis and activation of TA and that measuring their isotype, particularly IgM, and titers may be useful for determining the disease activity.

Although a single laboratory marker appears to be insufficient for assessing the disease activity in TA because it might reflect non-specific inflammation other than vasculitis, ESRs were more frequently elevated in the active disease group and their mean ESR was significantly higher than that of the stable disease group. Thus, it is interesting that IgM AECA titers correlated well with ESRs, but not with CRP levels. This discrepancy of disease activity with acute phase reactants had been also shown in those reported elsewhere (41-43) and is thought to be mainly caused by the fact that an elevation in the ESR is included as one criterion in the NIH criteria for active disease (1). Besides the correlation of IgM AECA with disease activity, IgG AECA titers

### Table III. Correlations between changes of IgM and IgG AECA titers and changes of laboratory parameters between before treatment and after treatment in patients with Takayasu’s arteritis.

<table>
<thead>
<tr>
<th></th>
<th>Δ IgM AECA</th>
<th></th>
<th>Δ IgG AECA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>r</td>
<td>p</td>
</tr>
<tr>
<td>Δ WBC count</td>
<td>0.19</td>
<td>NS</td>
<td>0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Δ ESR</td>
<td>0.54</td>
<td>0.003</td>
<td>0.26</td>
<td>NS</td>
</tr>
<tr>
<td>Δ CRP</td>
<td>0.11</td>
<td>NS</td>
<td>0.15</td>
<td>NS</td>
</tr>
</tbody>
</table>

Δ: The changes of each parameter measured between at active stage before treatment and at stable stage after treatment.

AECA: anti-endothelial cell antibodies, NS: not significant.
remained consistent throughout the disease course, and the changes in IgG AECA did not correlate with either disease activity or any of the laboratory parameters in this study. At present, it is unclear what the putative antigens of AECA are and which isotypes bind to them, and the pathogenic significance of each isotype of AECA is not defined yet. The reason for the difference between isotypes of AECA observed in the present study is obscure and, to clarify these subjects, further investigations for the putative target antigen and specificities of each isotype of AECA are needed.

The antiphospholipid antibodies can cause vascular thrombosis, which is one of the common features of TA (2). There have been a few studies mentioning the presence of antiphospholipid antibodies in patients with TA (22, 30, 44). Considering the pathologic findings of vascular inflammation combined with thrombosis, dysfunction of endothelial cell, and up-regulated markers for endothelial injury that are observed in TA (2, 45), these antibodies might have a pathogenic role in TA. However, these findings were not reproduced in another study (31). In our study, antiphospholipid antibodies were detected in few patients with TA and the positivity rates of these antibodies were not different from those in the controls. Neither their titers nor their isotype distributions did correlate with the disease activity. Thus, this finding suggests that vascular thrombosis in TA is not primarily caused by antiphospholipid antibodies.

There have been several evidences supporting a potential pathogenic role of autoantibodies for the arterial wall inflammation in patients with TA. The role of antiphospholipid antibodies is doubtful in this concern. However, our study suggests that AECA can serve as a possible participant in the pathogenesis of TA, and that their isotype distributions and titers may be used to follow the disease activity of TA. Further investigations for the putative target antigens would be helpful in determining a pathogenic role of AECA in TA.

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References
29. DEL PAPA N, GUIDALI L, SALA A et al.: Endothelial cells as target for antiphospho-


