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Antigenic Targets and Pathogenicity of Anti–Aortic Endothelial Cell Antibodies in Takayasu Arteritis

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Objective. **Anti–endothelial cell antibodies are considered to have an important role in the pathogenesis of Takayasu arteritis (TA). Previously, these antibodies were detected using human umbilical vein endothelial cells, which do not completely represent the antigenicity/functions of aortic endothelial cells, the specific targets in TA. To delineate the precise role of antigenic targets, we investigated such targets as well as the pathogenic mechanism of antibodies directed against aortic endothelial cells (AAECAs) in TA.**

Methods. **AAECAs were detected using a cellular enzyme-linked immunosorbent assay (ELISA), and their antigenic targets were detected by immunoblotting. AAECA-mediated induction of endothelial adhesion molecule expression and cytokine production was studied by ELISA, and apoptosis was studied using the TUNEL method.**

Results. **AAECAs were detected in 86% of patients with TA and in 9% of controls. Sera obtained from AAECA-positive patients with TA recognized a total of 9 antigens ranging in size from 18 kd to 200 kd, of which the 60–65-kd triplet was recognized most often. The aortic endothelial cell reactivity of Hsp60-absorbed sera was reduced by ~50% as compared with that of unabsorbed sera (mean ± SD 0.488 ± 0.08 versus 0.838 ± 0.116). Sera from AAECA-positive patients with TA, compared with sera from AAECA-negative patients with TA and that from controls, induced increased expression of E-selectin (mean ± SD 0.833 ± 0.063 versus 0.217 ± 0.081 and 0.221 ± 0.101 optical density [OD] units, respectively) and vascular cell adhesion molecule 1 (0.620 ± 0.144 versus 0.165 ± 0.005 and 0.177 ± 0.055 OD units, respectively) and increased production of interleukin-4 (IL-4) (6.8 ± 2.4 versus 1.2 ± 1.6 and 1.3 ± 2.5 pg/ml, respectively), IL-6 (24.3 ± 2.4 versus 4.5 ± 6.7 and 5.9 ± 5.1 pg/ml, respectively), and IL-8 (36.8 ± 10.3 versus 10.1 ± 6.7 and 7.8 ± 2.1 pg/ml, respectively). Sera from AAECA-positive patients with TA induced 29 ± 6% (median ± SEM) apoptosis of aortic endothelial cells.**

Conclusion. **Our data show that the AAECAs that are present in patients with TA are directed mainly against 60–65-kd antigen(s) and may cause vascular dysfunction by inducing expression of endothelial adhesion molecules, cytokine production, and apoptosis.**

Takayasu arteritis (TA) is a chronic granulomatous inflammatory arteriopathy that affects primarily large elastic arteries, mainly the aorta and its major branches. TA is characterized by stenosis, occlusion, or aneurysm of the involved arteries that eventually results in different clinical manifestations of the disease (1,2). TA has an autoimmune etiology, and immune-mediated dysfunction of aortic endothelium is considered to be the key event in the pathogenesis of the disease (3,4).

Immunoglobulin deposits in vascular lesions and the presence of circulating antibodies to human umbilical vein endothelial cells (HUVECs) (anti–endothelial cell antibodies [AECAs]) and their correlation with disease activity suggest that AECAs may be an important immune component involved in the development of TA (5–7). These antibodies may cause vascular dysfunction through multiple mechanisms, including upregulation of endothelial adhesion molecules and induction of cytochemokine production by endothelial cells, or through direct endothelial damage by apoptosis or other mechanisms (8). However, very limited informa-

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tion is available about the antigenic targets and precise pathogenic mechanism(s) of AECAs in TA. Furthermore, previous studies in TA have used HUVECs, which are fetal tissue–derived endothelial cells. Although HUVECs may have certain characteristics in common with aortic endothelial cells, they may not completely represent the antigenic and functional profile of those cells (9,10), which are specifically targeted in TA. Thus, in view of the type of vessel affected in TA, aortic endothelial cells would be the most clinically relevant target for studying the role of AECAs in TA. Until now, however, no study on AECAs directed against aortic endothelial cells (AAECAs) in TA has been available.

In the present study, we investigated antigenic targets of AAECAs and the induction of endothelial adhesion molecule expression, cytokine production, and apoptosis by these antibodies, in order to understand their pathogenic mechanism in TA.

PATIENTS AND METHODS

Patients. The study group comprised 35 patients with TA (25 women and 10 men; mean age 26 years [range 14–47 years]) and 21 age- and sex-matched healthy controls. The study was approved by the Institutional Ethics Committee of Sanjay Gandhi Postgraduate Institute of Medical Sciences. The diagnosis of TA was established on the basis of clinical, laboratory, and angiographic findings. All patients fulfilled the American College of Rheumatology 1990 criteria for a diagnosis of TA and had angiographically proven disease (11). The disease activity of the patients was determined according to previously described criteria (12). Accordingly, 14 patients had active TA, and 21 patients had inactive TA. The patients with active TA were assigned to an immunosuppressive regimen consisting of 1 mg/kg/day of prednisolone and 2 mg/kg/day of azathioprine. Once disease became inactive, patients received maintenance dosages of prednisolone (5–10 mg/day) and azathioprine (2 mg/kg/day). Immunosuppressive therapy was given for a period of 2 years.

After informed consent was obtained, 5 ml of venous blood was collected from each individual, and isolated serum was stored at −80°C until analyzed. Sera from all patients and controls were used for detecting AAECAs, whereas 10 high-titer AAECA-positive TA sera, 5 AAECA-negative TA sera, and 12 control sera (including 2 AAECA-positive sera) were used for immunoblotting and functional studies.

Culture of aortic endothelial cells. Normal human aortic endothelial cells and cell culture reagents were procured from Cambrex Bioscience (Walkersville, MD). Cells were cultured under standard conditions, and subcultures of passages 4–6 were used for the experiments.

Detection of AAECAs. AAECAs were detected by cellular enzyme-linked immunosorbent assay (ELISA), as described previously (6). Briefly, aortic endothelial cells were seeded in 96-well culture plates to obtain a monolayer of cells. The cells were washed twice with phosphate buffered saline (PBS) and fixed with 0.2% glutaraldehyde and 0.2% Triton X-100 for 20 minutes. After blocking nonspecific binding sites with 5% bovine serum albumin (BSA), test serum diluted 1:100 was added at 100 μl/well and incubated for 2 hours at 37°C. The bound antibodies were detected with goat anti-human IgG peroxidase-conjugated antibodies (Dako, Kyoto, Japan), using tetramethylbenzidine substrate. Washings between steps were performed with PBS containing 0.5% BSA. Optical density (OD) was measured at 450 nm in an automated ELISA reader (Spectra; Tecan, Grödig, Austria). OD values greater than the mean + 2 SD value of the normal controls were considered positive.

Characterization of antigenic targets of AAECAs. The antigenic targets of AAECAs were characterized by Western blotting, as described elsewhere (13). A monolayer of aortic endothelial cells washed twice with PBS was harvested by scraping with a rubber scraper. The cells were mixed with lysis buffer and incubated for 20 minutes on ice to ensure homogeneous solubilization. The lysate was spun at 12,000 revolutions per minute for 15 minutes, and the supernatants obtained were subjected to electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide gels. Proteins were electrotransferred from the gels onto a polyvinylidene difluoride membrane. Nonspecific binding sites on the membrane were blocked with 5% BSA for 2 hours at 37°C. The membrane was cut into strips and incubated overnight with serum samples diluted 1:10. The strips were then incubated for 90 minutes with goat anti-human IgG peroxidase-conjugated antibody (Dako), and color was developed with diaminobenzidine (Bio-Rad, Hercules, CA).

Determination of absorption of Hsp60 activity of sera. To determine whether Hsp60 is one of the target antigens of AAECAs on aortic endothelial cells, we performed an absorption study using sera from 10 patients with TA that contained high titers of AAECAs. The sera were diluted 1:100 and incubated for 1 hour at 37°C in 5 successive wells coated with Hsp60 (10 μg/ml), and the endothelial reactivity of these absorbed and unabsorbed sera was determined by cellular ELISA using aortic endothelial cells, as described above.

Detection of AAECA-induced E-selectin and vascular cell adhesion molecule 1 (VCAM-1) expression. Aortic endothelial cells grown in 96-well culture plates were incubated with test sera (diluted 1:10) for 24 hours at 37°C. Thereafter, culture was terminated, and cellular ELISA (as previously described for detection of AAECAs) was performed for detection of AAECA-induced E-selectin and VCAM-1 expression, by using peroxidase-conjugated mouse anti-human E-selectin/VCAM-1 antibodies (R&D Systems, Minneapolis, MN).

Detection of AAECA-induced cytokine production. Aortic endothelial cells grown in 96-well culture plates were incubated with test sera (diluted 1:10) for 24 hours at 37°C. The supernatant from individual wells was collected to evaluate cytokine production by the cells. The detection of cytokines (interleukin-4 [IL-4], IL-6, IL-8, IL-10, and tumor necrosis factor α [TNFα]) in the supernatant was performed using commercial kits (BD Biosciences, San Jose, CA), according to the manufacturer's instructions.

Detection of AAECA-induced apoptosis. Aortic endothelial cells grown in 24-well culture plates were incubated with test sera (diluted 1:10) for 24 hours, and apoptosis in the harvested cells was detected by the TUNEL method, using the ApoDIRECT flow cytometry kit (BD Biosciences) (14). Cells

were analyzed in a single-parameter histogram showing fluorescein isothiocyanate–dUTP on the x-axis and the relative cell number on the y-axis, using CellQuest software (CellQuest, Largo, FL). Cells in the M1 gate were regarded as being negative cells, whereas the presence of apoptotic cells was demonstrated in the M2 gate.

Statistical analysis. Statistical analysis was performed using the Mann-Whitney U test for the comparison of means and Fisher's exact test for the analysis of frequency. Data are expressed as the mean ± SD or median ± SEM. *P* values (2-tailed) less than 0.05 were considered significant.

RESULTS

Prevalence of AAECAs. AAECAs were observed in the sera of 30 (86%) of 35 patients with TA compared with 2 (9%) of 21 healthy controls ($P = 0.001$). The levels of AAECAs were also significantly higher in patients compared with healthy controls (0.819 ± 0.429 versus 0.288 ± 0.111; $P = 0.001$). However, there was no difference in the prevalence of AAECAs in patients with active TA (12 [86%] of 14) and those in whom TA was inactive (18 [86%] of 21). Similarly, no difference in the levels of AAECAs was observed between patients with active TA and those with inactive TA (0.952 ± 0.524 versus 0.735 ± 0.332; $P = 0.219$) (Figure 1).

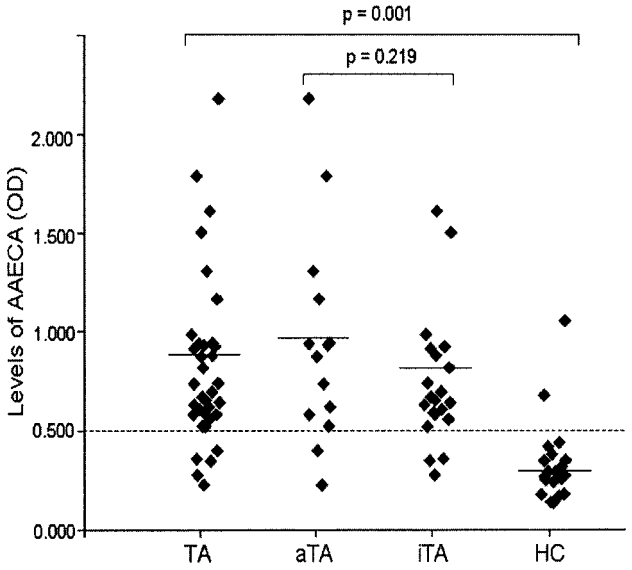

Figure 1. Dot plot showing levels of anti–aortic endothelial cell antibodies (AAECAs) in all patients with Takayasu arteritis (TA), patients with active TA (aTA), patients with inactive TA (iTA), and healthy controls (HC). Each dot represents the data for an individual subject. The solid horizontal lines show the means. The broken horizontal line represents the cutoff limit (mean + 2 SD of values in the control group). OD = optical density.

Table 1. Molecular weights and frequency of antigenic targets recognized by AAECAs in patients with TA and healthy controls*

Group/antigenic target	Frequency of recognition
AAECA-positive patients (n = 10)	
18 kd	2/10
21 kd	3/10
27 kd	5/10
28 kd	4/10
32 kd	6/10
44 kd	1/10
60–65 kd	10/10
80 kd	3/10
200 kd	6/10
AAECA-negative patients (n = 5)	
NA	NA
Healthy controls (n = 12)	
27 kd and 28 kd	1/12

* AAECAs = anti–aortic endothelial cell antibodies; TA = Takayasu arteritis. NA = not applicable.

Antigenic targets of AAECAs. The molecular weights of proteins recognized by AAECAs from different groups of study subjects are shown in Table 1, and their representative immunoblots are shown in Figure 2. Sera obtained from AAECA-positive patients reacted with 3–5 of 9 aortic endothelial cell antigens ranging from 18 kd to 200 kd. The most common reactivity was against a triplet of bands ranging from 60 kd to 65 kd. No protein bands were recognized by AAECA-negative TA

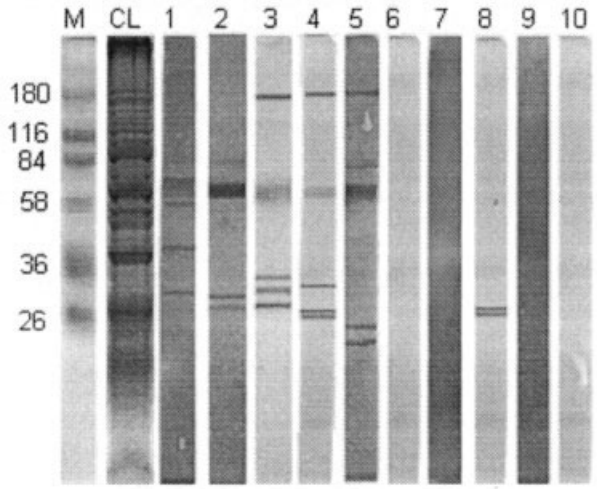

Figure 2. Representative immunoblots showing the molecular weights of different protein bands recognized by sera from anti–aortic endothelial cell antibody (AAECA)–positive patients with Takayasu arteritis (TA) (lanes 1–5), AAECA-negative patients with TA (lanes 6 and 7), and healthy controls (lanes 8–10). M = molecular weight marker; CL = cell lysate of aortic endothelial cells.

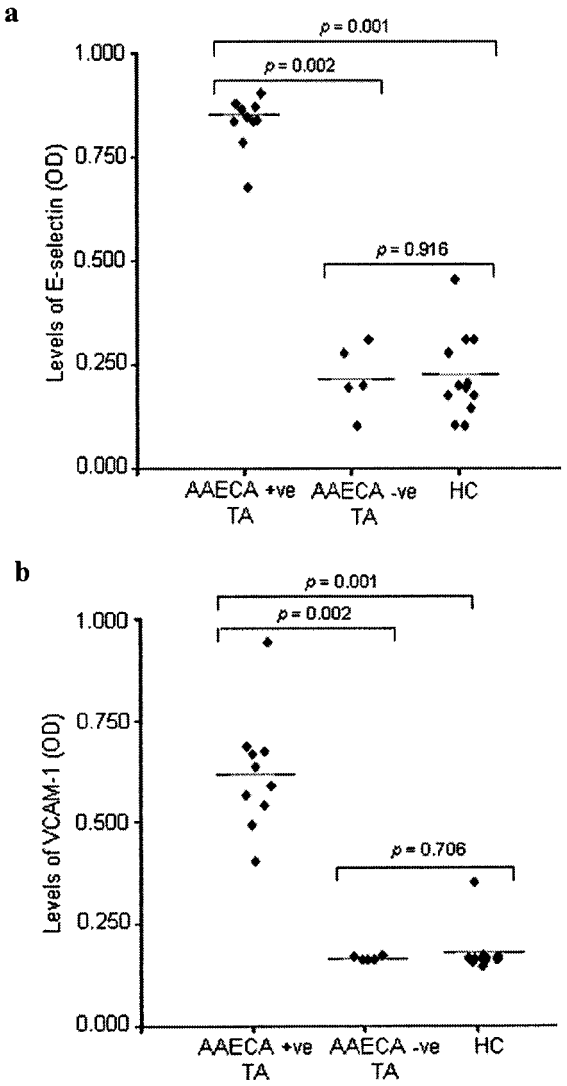

Figure 3. Dot plots showing expression of E-selectin (**a**) and vascular cell adhesion molecule 1 (VCAM-1) (**b**) by aortic endothelial cells stimulated with sera from anti–aortic endothelial cell antibody (AAECA)–positive patients with Takayasu arteritis (TA), AAECA-negative patients with TA, and healthy controls (HC). Horizontal lines represent the means. OD = optical density.

sera. Similarly, sera from healthy controls did not recognize any of the protein bands, with the exception that 1 of the AAECA-positive sera showed reactivity to a doublet corresponding to 27-kd and 28-kd antigens.

Absorption of Hsp60 activity of sera. The aortic endothelial cell reactivity of Hsp60-absorbed sera was reduced by ~50% as compared with that of unabsorbed sera (0.488 ± 0.08 versus 0.838 ± 0.116; $P = 0.009$).

AAECA-induced E-selectin and VCAM-1 expression. All AAECA-positive TA sera, but none of the AAECA-negative TA sera or control sera up-regulated

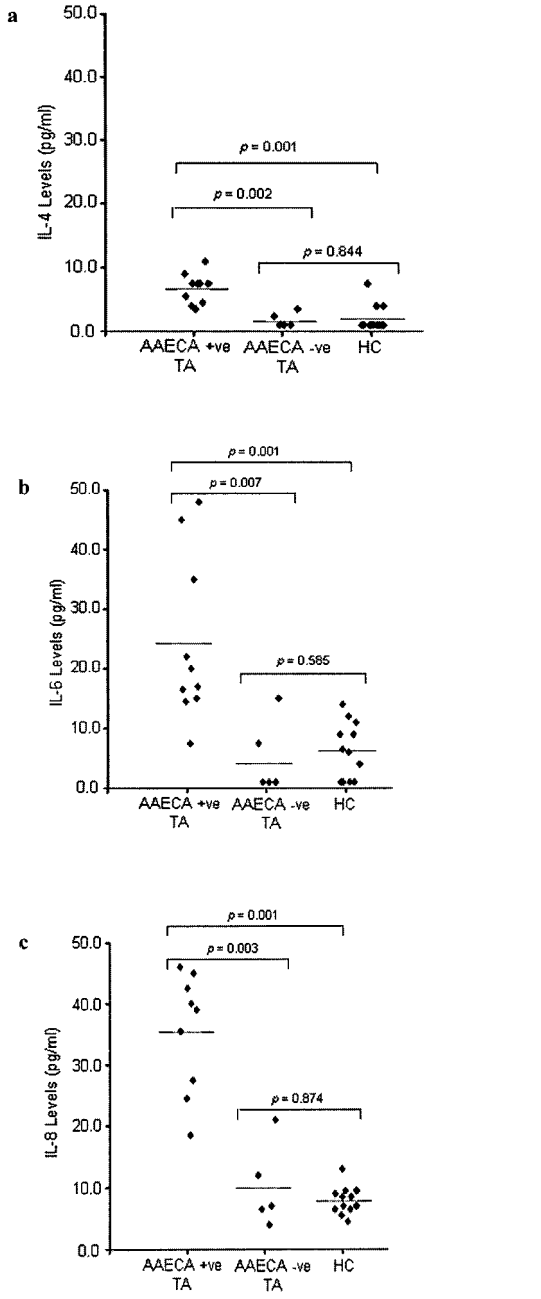

Figure 4. Dot plots showing production of interleukin-4 (IL-4) (**a**), IL-6 (**b**), and IL-8 (**c**) by aortic endothelial cells stimulated with sera from anti–aortic endothelial cell antibody (AAECA)–positive patients with Takayasu arteritis (TA), AAECA-negative patients with TA, and healthy controls (HC). Horizontal lines represent the means.

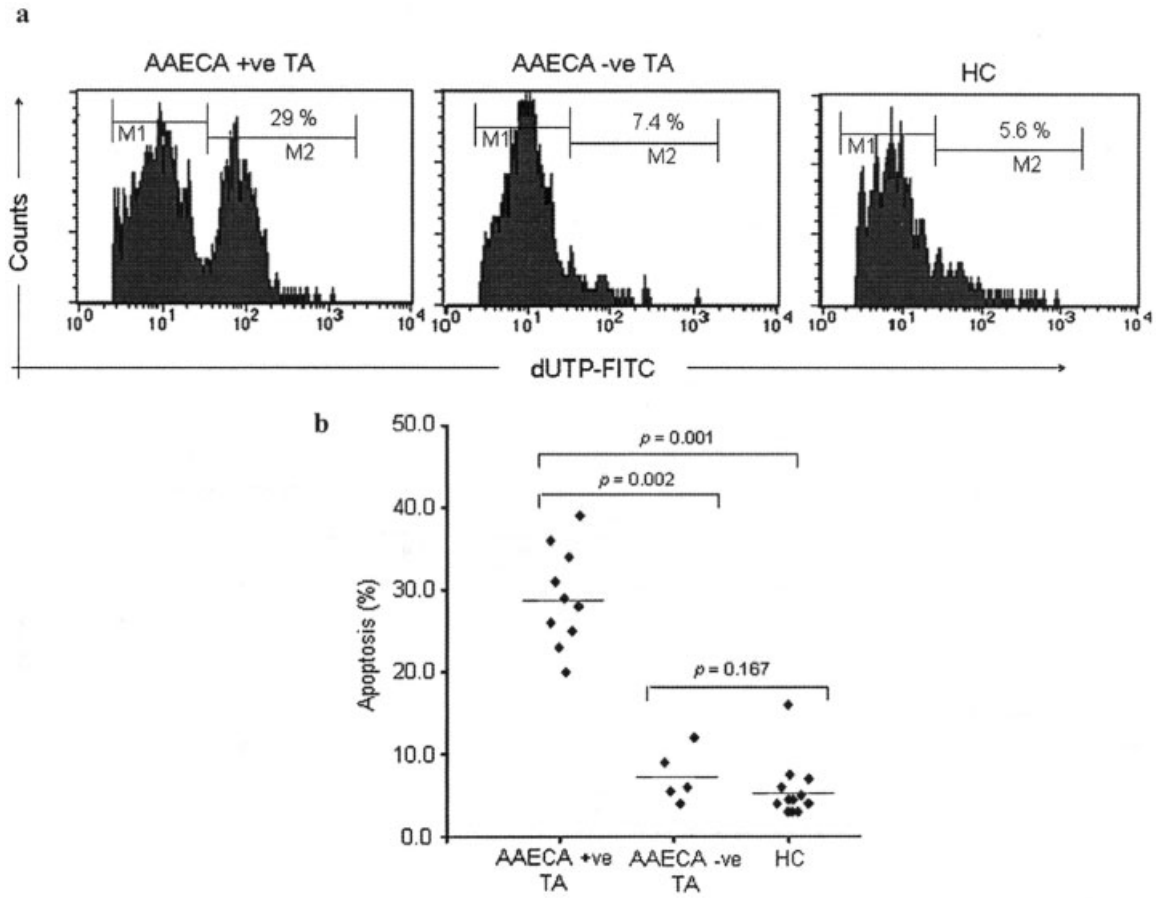

Figure 5. Representative flow cytometry histograms (**a**) and dot plots (**b**) showing endothelial cell apoptosis induced by sera from anti–aortic endothelial cell antibody (AAECA)–positive patients with Takayasu arteritis (TA), AAECA-negative patients with TA, and healthy controls (HC). Percentages in **a** indicate the proportion of apoptotic cells. Horizontal lines in **b** represent the medians. FITC = fluorescein isothiocyanate.

the expression of E-selectin and VCAM-1 by aortic endothelial cells. Sera from AAECA-positive patients with TA, compared with sera from AAECA-negative patients with TA and healthy controls, induced significantly increased expression of E-selectin (0.833 ± 0.063 versus 0.217 ± 0.081 and 0.221 ± 0.101 OD units, respectively; $P = 0.002$ and $P = 0.001$, respectively) and VCAM-1 (0.620 ± 0.144 versus 0.165 ± 0.005 and 0.177 ± 0.055 OD units, respectively; $P = 0.002$ and $P = 0.001$, respectively) (Figure 3).

AAECA-induced cytokine production. Sera from AAECA-positive patients with TA, compared with sera from AAECA-negative patients with TA and healthy controls, significantly increased cytokine production by aortic endothelial cells (for IL-4, 6.8 ± 2.4 versus 1.2 ± 1.6 and 1.3 ± 2.5 pg/ml, respectively [$P = 0.002$ and $P = 0.001$, respectively]; for IL-6, 24.3 ± 2.4 versus 4.5 ± 6.7 and 5.9 ± 5.1 pg/ml, respectively [$P = 0.007$ and $P = 0.001$, respectively]; and for IL-8, 36.8 ± 10.3 versus 10.1 ± 6.7 and 7.8 ± 2.1 pg/ml, respectively [$P = 0.003$ and $P = 0.001$, respectively]) (Figure 4). In contrast, production of IL-10 and TNFα by aortic endothelial cells was undetectable in all of the study groups.

AAECA-induced apoptosis. All AAECA-positive TA sera induced apoptosis in aortic endothelial cells, whereas no apoptosis of cells was observed in any of the AAECA-negative TA sera and control sera, with the exception of AAECA-positive control sera, which induced 16% apoptosis. The magnitude of apoptosis in aortic endothelial cells induced by the sera of AAECA-positive patients with TA was a median ± SEM 29 ± 6% (Figure 5). Baseline apoptosis in untreated aortic endothelial cells was observed to be ~6%.

DISCUSSION

Our study shows that most patients with TA have circulating AAECAs, which are directed predominantly to a triplet of aortic endothelial cell antigens ranging in size from 60 kd to 65 kd. These AAECAs induce adhesion molecule expression and inflammatory cytokine production by aortic endothelial cells and cause apoptosis of aortic endothelial cells. To our knowledge, this study is the first to explore the prevalence, antigenic targets, and pathogenicity of AAECAs in TA.

Previous studies using HUVECs as antigenic substrate have shown the prevalence of AECAs in a larger proportion of patients with active TA. We previously reported the overall prevalence of AECAs to be 33% in patients with TA, 62% in patients with active TA, and 18% in patients with inactive TA, which demonstrates their correlation with disease activity (6). Similarly, other investigators (7) have reported that 95% of patients with active TA have AECAs (7). In the present study, we observed the overall prevalence of AAECAs to be 86%, which is in the range of the reported prevalence of AECAs in patients with active TA. However, unlike the above-mentioned studies on AECAs, in our study we observed the prevalence of AAECAs to be similar in patients with active TA and in those with inactive TA, which shows no correlation with disease activity. One important reason for this could be the nonspecific nature of existing markers of disease activity; thus, the patients who appear to have clinically inactive disease may actually have histologically active disease, as has been reported previously (15). Another reason could be that AAECAs and AECAs are directed to different epitopes of endothelial cells, and that these epitopes against which AAECAs are directed are also expressed during the inactive stage of disease.

The presence of circulating AAECAs in most of the patients with TA prompted us to investigate the antigenic targets against which these antibodies may be directed. We performed immunoblotting, using aortic endothelial cell lysate as antigen, and observed that 3–5 of the 9 protein bands ranging in size from 18 kd to 200 kd were recognized by AAECAs. Of these, a triplet of bands ranging in size from 60 kd to 65 kd was uniformly recognized by AAECAs from all patients. These immunoblotting results indicate that the AAECAs that are present in patients with TA are a heterogeneous group of antibodies directed against multiple protein molecules of aortic endothelial cells and may have multiple roles in the disease, depending on the antigenic target against which they are directed.

Similar to our findings, a previous immunoprecipitation study on a single patient with TA showed a broad range of HUVEC antigens (20–175 kd) recognized by polyclonal AECAs. Of these HUVEC antigens, 3 predominant bands in the range of 60–65 kd were strongly recognized by 4 of 6 monoclonal AECAs generated from the same patient and by polyclonal AECAs as well (16). Taken together, the findings of that study and ours suggest that the most common endothelial antigens recognized by AAECAs or AECAs may be Hsp60 or other homologous endothelial antigens. This was also confirmed by our absorption study, which showed a significant reduction in aortic endothelial cell reactivity of Hsp60-absorbed sera. Increased expression of Hsp60/65 in the aortic lesions of patients with TA (17), and our previous study demonstrating a high prevalence of anti-Hsp60/65 antibodies as well as T cell reactivity to these heat-shock protein antigens in TA, lend further support to this hypothesis (18).

AECAs represent an extremely heterogeneous group of autoantibodies and are classified broadly as antibodies to microvascular and macrovascular endothelial cells, depending on the type of vasculature targeted (19). Functionally, they may be pathogenic or nonpathogenic. The pathogenic antibodies may have activating, apoptotic/cytotoxic, or both types of effects on endothelial cells (20). In addition, some AECAs are directed toward different procoagulant and anticoagulant endothelial cell molecules such as thrombomodulin, heparin, and glycoprotein IV (CD36), and induce thrombosis by modulating the properties of these molecules (21–23). Such AECAs are observed particularly in the setting of microvasculopathies such as thrombotic thrombocytopenic purpura and scleroderma (21,23). Similarly, autoantibodies present in the sera of vasculitic mice (i.e., a murine model of vasculitis) that react with smooth muscle cells and AECAs with such cross-reactivity may cause aneurysm by inducing damage to smooth muscle cells (24). Such AECAs may be observed in macrovasculopathies such as TA. Thus, depending on the antigenic targets and the type of vasculature involved, AECAs may have different pathogenic roles in different autoimmune diseases.

To determine whether the AAECAs that we observed have pathogenic relevance in TA, we investigated the activating potential of these antibodies in terms of the induction of adhesion molecule expression and cytokine production by aortic endothelial cells. Our adhesion molecule data showed that AAECAs from all of the patients induced endothelial up-regulation of E-selectin and VCAM-1. Corroborating our data, most

of the monoclonal AECAs generated from a single TA patient were also shown to induce expression of E-selectin and VCAM-1 on HUVECs (16). Thus, AAECA-induced up-regulation of adhesion molecules by aortic endothelial cells may have a critical role in adhesion and in the arterial recruitment of circulating inflammatory leukocytes, which in turn may cause tissue damage through various pathways.

In the cytokine studies, we observed that AAECAs from all of the patients with TA induced IL-4, IL-6, and IL-8 production by aortic endothelial cells. Previous studies in TA showing AECA-induced production of IL-6 by HUVECs and histologic expression of IL-6 messenger RNA (mRNA) in aortic tissues validate our findings of AAECA-induced production of IL-6 in patients with TA (16,25). There is no study on AECA-induced IL-8 production in TA, but studies in Wegener's granulomatosis have shown AECA-induced production of IL-8 by HUVECs (26,27). Furthermore, there are no available studies on AECA-induced production of IL-4 in TA or other vasculitides.

Production of these inflammatory cytokines by AAECA-stimulated aortic endothelial cells may have important implications in TA. IL-4 is important in the up-regulation of VCAM-1 and chemokines such as monocyte chemotactic protein 1 by endothelial cells. Moreover, it may cause apoptosis of endothelial cells through the caspase 3–dependent pathway and may have direct involvement in the induction of disease (28,29). IL-6, in addition to up-regulating endothelial adhesion molecules, induces humoral and cellular immune responses by mediating antibody production by B cells and the cytotoxic activity of T cells and natural killer cells, the main inflammatory cells involved in TA (30,31). IL-8 is a potent chemokine and may have an important role in the recruitment of neutrophils as well as mononuclear cells at the site of inflammation (32).

Thus, AAECA-induced production of IL-4, IL-6, and IL-8 may, indeed, be the initial event that eventually leads to arterial damage through different mechanisms. AECA-induced production of TNFα and IL-10 by endothelial cells has not been reported in any vasculitic disorder, including TA. We also observed no production of TNFα and IL-10 by AAECA-stimulated aortic endothelial cells, which indicates that these (endothelial-derived) cytokines may have no role in the immunopathogenesis of TA. Results of previous studies in TA that showed very weak expression of TNFα mRNA in arterial lesions also validate our findings (25).

We further investigated the apoptosis-inducing potential of the observed AAECAs, in order to determine whether these antibodies have a direct pathogenic effect on aortic endothelial cells. It was observed that AAECAs from all of the patients with TA induced apoptosis of aortic endothelial cells, and the degree of apoptosis ranged from 20% to 39%. No data are available on AECA-mediated apoptosis or the cytotoxicity of endothelial cells in TA, with the exception of our previous study (33), which showed AECA-mediated complement-dependent cytotoxicity of HUVECs. In another study, we reported that >50% of AECAs possess anti–annexin V activity, a potent inducer of endothelial cell apoptosis, which supports the apoptosis-inducing potential of AAECAs (12). It has also been demonstrated that the binding of AECAs to endothelial cells causes externalization of phosphatidylserine and induces apoptosis of endothelial cells (34,35). Because 60-kd endothelial antigen (Hsp60)–reactive AECAs have been shown to cause apoptosis of endothelial cells (36,37), it is quite likely that the AAECAs that we observed to be directed against 60–65-kd aortic endothelial cell antigens may be a subgroup of these antibodies with apoptosis-inducing potential. The results of those studies, including the present one, collectively suggest that the AAECAs that we observed may cause apoptotic damage of arterial endothelium and thus may have a direct role in the pathogenesis of TA.

In conclusion, we have shown that most patients with TA possess circulating AAECAs that are directed mainly against 60–65-kd endothelial cell antigens and may have a pathogenic role in the disease via up-regulation of adhesion molecule expression, inflammatory cytokine production, and direct apoptotic damage of arterial endothelium. Further studies in this area would be important for a greater understanding of the etiopathogenesis of TA.

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