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Detection of Antiendothelial Cell Antibodies in Patients with Connective Tissue Diseases by Flow Cytometry and Their Relation to Endothelial Cell Activation

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Summary

Antiendothelial cell antibodies (AECA) have been detected by flow cytometry analysis in 23 out of 80 patients with connective tissue diseases. Ten out of 19 serum samples from patients with systemic lupus erythematosus (SLE) were positive. These antibodies were not detectable in healthy donors. We examined the capacity of serum samples to induce endothelial cell activation by modulating cell adhesion molecule expression on human umbilical vein endothelial cells. We found that sera from both AECA-positive and AECA-negative patient groups induced a significantly higher expression of E-selectin compared to healthy controls ($P<0.05$). There were no differences in the ICAM-1 on VCAM-1 expression. Our data suggest that increased E-selectin expression in activated endothelium in patients with various connective tissue disorders is not related to the production of AECA.

Key words

Antiendothelial cell antibodies • Adhesion molecules • Expression • Flow cytometry

Introduction

Antiendothelial cell antibodies (AECA) represent a heterogeneous group of antibodies directed against a variety of antigenic determinants on endothelial cells. They were first noted more than 20 years ago, during an immunohistochemical study of kidney biopsy specimens from animals and humans. AECA occur during various autoimmune diseases, including systemic lupus erythematosus (SLE), scleroderma, Wegener´s granulomatosis, mixed connective tissue diseases, the hemolytic uremic syndrome, Sjogren´s syndrome, rheumatoid arthritis, vasculitis, lupus nephritis, Kawasaki disease and progressive systemic sclerosis (Frampton *et al.* 1990, D´Cruz *et al.* 1991, Westphal *et al.* 1994, Moroni *et al.* 2001).

The vascular endothelium is a metabolically active layer of cells that forms an interface between the bloodstream and tissues. The position of these cells indicates their crucial importance in modulating and directing biological responses, including the regulation of hemostasis and trafficking of leukocytes to inflammatory sites. The immunoglobulin deposition along the human vascular endothelium correlated with the accumulation of lymphocytes within the vessel wall of postcapillary venules, suggest a possible role for AECA in leukocyte homing and recirculation. The endothelium is readily accessible to these elements of the immune system and

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constitutively express epitopes that can be targeted by alloantibodies or autoantibodies. Additionally, these cells form a highly metabolically active tissue that responds to cellular mediators or microorganisms, and they may up- or downregulate the antigenic determinants or express novel ones during this process (Ferraro *et al.* 1990, Carvalho and Savage 1997, Cockwell *et al.* 1997).

AECA may play a pathophysiological role by activating endothelial cell resulting in the upregulation of the cell adhesion molecule (CAM) expression, secretion of chemoattractants (MCP-1, IL-8) and cytokines such as IL-1 and IL-6. Furthermore, they sustain leukocyte adherence to the endothelium, and an antibody subset may be capable of initiating endothelial cell apoptosis. AECA are apparently distinct from other autoantibodies (ANCA or anti-DNA). A number of methods have been used to detect AECA including indirect immuno-fluorescence, cell enzyme-linked immunosorbent assay, radioimmunoassay and Western blotting. Due to the lack of assay standardization, the incidence of AECA varies between investigators. The detection of these antibodies may be valuable in following pathological activity. Further characterization of putative antigens is needed for understanding their pathophysiological role (Hashemi *et al.* 1987, Heurkens *et al.* 1991, Carvalho *et al.* 1996, 1999, Renaudineau *et al.* 1999, Griesmacher and Peichl 2001).

The aim of this paper is to examine the prevalence of antibodies directed against human umbilical vein endothelial cells (HUVEC) in the sera of patients with connective tissue diseases. We have also studied the relationship of these antibodies to CAM expression (ICAM-1, VCAM-1, E-selectin).

Methods

Patients and controls

We studied 80 patients with connective tissue diseases (mean age 45 years; 22 men and 58 women), including 19 systemic lupus erythematosus (SLE), 2 systemic sclerosis, 1 polyarteritis nodosa, 12 other necrotizing vasculopathies, and 46 other systemic connective tissue afflictions, each of whom fulfilled the diagnostic criteria of the American Rheumatism Association (Fauci *et al.* 1978, Tan *et al.* 1982, Medsger and Steen 1996). These patients were recruited from the Department of Clinical Immunology at the Institute of Preventive and Clinical Medicine, Bratislava. The sera obtained from 52 normal healthy subjects (mean age 27 years; 25 men and 27 women) were used as the control group. All subjects gave their informed written consent.

Isolation and culture of endothelial cells

HUVEC were isolated as previously described (Jaffe *et al.* 1973) and cultured on 1 % gelatine matrix in RPMI 1640 medium supplemented with 15 % fetal bovine serum (BioWhittaker, Inc.), 1 % L-glutamine, antibiotics, heparin and 30 μg/ml endothelial cell growth supplement (Sigma Chemical Co.). The confluent fourth passage was used for the experiments.

Detection of AECA

Detection of AECA was performed according to the modified method of Westphal *et al.* (1994). HUVEC were detached from culture dishes non-enzymatically, and incubated in a suspension with undiluted test serum ($1x10^5$ HUVEC/100 μl sera / tube) at 4 °C for 2 h. After removing unbound antibodies by washing with 1 % bovine serum albumin in phosphate-buffered saline (PBS), cells were stained with fluorescein-conjugated $F(ab')_2$ fragment goat anti-human immunoglobulin (IgG) (H+L) (Coulter-Immunotech). Cells were subsequently analyzed in a flow cytometer (Coulter Epics xl) running under System II software (Coulter). The results were expressed as the percentage of AECA-binding activity .

The following controls: HUVEC control (culture medium), negative control (pooled serum from 20 healthy donors) and a laboratory positive control (highly positive sample from a patient with graft rejection after renal transplantation) were used in each assay. A level of more than 10 standard deviations above the average of the healthy controls (n = 50) was considered as positive.

Analysis of CAM expression by flow cytometry

HUVEC (1×10^5) were incubated in the presence or in the absence (HUVEC control) of 100 μl undiluted test serum at 37 °C in 5 % CO_2. The incubation period in preliminary experiments was found to be 24 h for ICAM-1 and 12h for VCAM-1 and E-selectin. The appropriate amounts of unbound monoclonal antibodies anti-ICAM, anti-VCAM-1, anti-E-selectin or negative control IgG1 and IgG2a (Becton Dickinson) were used accordingly. The subsequent steps included washing in PBS and incubation with fluorescein isothiocyanate-labeled goat anti-mouse secondary antibody (Becton Dickinson) for 30 min at room temperature. CAM expression was examined by flow cytometry analysis. Data are expressed as the percentage of positive cells after subtraction of fluorescence background.

Statistical analysis

The Fisher´s exact, and Wilcoxon Mann-Whitney tests were used where appropriate for determining the statistical significance. $P<0.05$ values were regarded as significant.

Results

Detection of antiendothelial cell antibodies

Sera from patients with connective tissue afflictions and healthy donors were tested for the presence of AECA. All sera from healthy subjects were negative, whereas 23 out of 80 (29 %) of the patient sera were positive ($P<0.00001$). Ten of 19 serum samples from SLE patients were positive (53 %) (Table 1).

Table 1. Antiendothelial cell antibodies in patients with connective tissue diseases

	All patients	SLE patients	Control group
Negative	57/80 (71 %)	9/19 (47 %)	52/52 (100 %)
Weakly positive	16/80 (20 %)	8/19 (42 %)	
Positive	7/80 (9 %)	2/19 (11 %)	

Analysis of endothelial cell activation

In order to determine whether patient and/or healthy donors serum samples might modulate endothelial cell expression, 14 healthy subjects, 14 AECA-positive and 14 AECA-negative patient sera were tested on HUVEC. Figure 1 shows that the treatment of HUVEC with sera was accompanied by increased expression of E-selectin in both AECA-positive and AECA-negative patient groups in comparison to healthy controls ($P<0.05$). No differences in the induction of ICAM-1 and VCAM-1 expression were found in all tested groups.

Discussion

The present study was undertaken to investigate the presence of AECA in sera of patients with connective tissue diseases. We showed that 29 % of all patients were AECA-positive, whereas no positively reacting serum was found in the healthy group. We suggest that the highest incidence of positivity is present in the group of SLE patients (53 %). Our data confirm previous reports that AECA have been observed in various autoimmune diseases (Cines *et al.* 1984, Vismara *et al.* 1988, Brasile *et al.* 1989, Heurkens *et al.* 1991, Carvalho *et al.* 1996, Lee *et al.* 1999, Griesmacher and Peichl 2001). The role of AECA is still not completely understood. Some authors have shown that AECA activate endothelial cells and thus initiate autoimmune vasculitic disorders (Brasile *et al.* 1989, Carvalho *et al.* 1996, Renaudineau *et al.* 1999, Muller Kobold *et al.* 1999), whereas others have postulated AECA-induced apoptosis in the endothelium (Bordron *et al.* 1998).

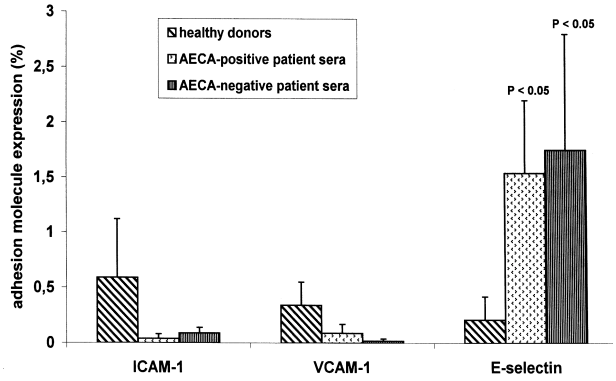

Fig. 1. *Effect of sera on the expression of endothelial cell adhesion molecules. HUVEC were treated with sera from healthy subjects (n=14), AECA-positive (n=14) and AECA-negative patients (n=14). ICAM-1, VCAM-1 and E-selectin expression was analyzed by flow cytometry. The zero level represents HUVEC background. Each bar represents the average ± S.E.M. $P<0.05$ compared with healthy donors.*

Endothelial cell activation is defined as "quantitative changes" in the level of specific gene product expression (i.e. proteins) that in turn endow endothelial cell with a new capacity that cumulatively allows them to perform new functions (Cockwell *et al.* 1997). The target antigens for AECA are not yet defined, but it is clear that they are likely to be complex and to differ in different diseases (Carvalho *et al.* 1999). The endothelial cell antigens can be constitutively expressed and/or modulated by cytokines, or cryptic. In addition, antigen determinants for AECA may also concern molecules that adhere to the endothelium. An alternative mechanism by which AECA could act as a trigger in the pathogenesis of some diseases is complement cytotoxicity and/or antibody-dependent cellular

cytotoxicity (Cines *et al.* 1984, Raife *et al.* 1999, Griesmacher and Peichl 2001, Tripathy *et al.* 2001). More recently, speculation about the mechanism of vascular damage has focused on the enhanced expression of CAM by the endothelium (notably ICAM-1, VCAM-1 and E-selectin) (Carvalho *et al.* 1996, Youinou *et al.* 1999).

Previous reports (Simantov *et al.* 1995, Cockwell *et al.* 1997, Lucchiari *et al.* 2000) have shown that the serum or IgG from patients with vascular, renal and others inflammatory diseases can modulate CAM expression or the adhesive phenotype of cultured endothelial cells. Some investigators found that endothelial cell activation can be induced by AECA. Phenotype changes of the endothelium can be due in part to the induction of IL-1 synthesis, and other mediators (IL-1, -2, -4, -6, -8, MIP-1) (Carvalho *et al.* 1996, 1999, Renaudineau *et al.* 1999, Raife *et al.* 1999, Youinou *et al.* 1999). Our data suggest that both AECA-positive and AECA-negative patient sera induce significantly higher E-selectin expression than healthy donors. These findings agree with a previous study (Muller Kobold *et al.* 1999), however, we have not found any relationship between CAM expression and the content of AECA.

In summary, the results reported herein strengthen the hypothesis that endothelial cell activation, such as increased E-selectin expression, is a feature of connective tissue diseases, but there is no evidence that these changes are the result of raised levels of AECA.

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