Anti-endothelial cell antibodies in mixed connective tissue disease: Frequency and association with clinical symptoms

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Abstract

Objective

Anti-endothelial cell antibodies (AECA) have been described in a number of systemic autoimmune-inflammatory diseases. However, little is known about the relationship of AECA with mixed connective tissue disease (MCTD).

Methods

Using an ELISA, the presence of AECA was evaluated in the sera of 33 patients with MCTD and of 30 healthy subjects as controls. Serum levels of AECA were correlated with clinical activity, as well as the existence of various organ manifestations.

Results

Significantly increased AECA production was observed in MCTD patients (OD = 0.337 ± 0.193) compared to controls (OD = 0.136 ± 0.065). In addition, patients with active MCTD exerted significantly elevated serum AECA levels (OD = 0.487 ± 0.090) than did patients with inactive MCTD (OD = 0.135 ± 0.040) or controls. MCTD patients with pulmonary hypertension had a tendency of increased serum AECA levels (OD = 0.452 ± 0.080) compared to patients without this manifestation (OD = 0.307 ± 0.039). Sera of MCTD patients with AECA concentrations higher or lower than the mean serum AECA level in controls+2SD (OD = 0.266) were considered as $AECA^{high}$ (n = 19/33) and $AECA^{low}$ (n = 14/33), respectively. Interestingly, all patients with active disease had $AECA^{high}$, while all inactive MCTD patients had $AECA^{low}$ sera. IgG purified from ten MCTD sera (OD = 0.415 ± 0.290) showed a tendency to up-regulate E-selectin expression on cultured human umbilical vein endothelial cells (HUVEC) compared to IgG from control sera. In addition, $AECA^{high}$ MCTD sera exerted significantly increased stimulatory effect on endothelial E-selectin expression (OD = 0.651 ± 0.190) compared to $AECA^{low}$ (OD = 0.178 ± 0.110) or control sera (OD = 0.131 ± 0.080).

Conclusion

AECA may activate endothelial cells by the up-regulation of E-selectin expression and thus may be implicated in the pathogenesis of MCTD. Furthermore, serum AECA may be a useful marker of endothelial activation and clinical activity in this disease.

Key words

Anti-endothelial cell antibodies, mixed connective tissue disease, disease activity.

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Introduction

Mixed connective tissue disease (MCTD) is a systemic autoimmune disease with major clinical features including Raynaud's phenomenon, swelling of the hands, arthritis, esophageal hypomotility, myositis, and pulmonary involvement, such as interstitial fibrosis and obliterative vasculopathy with pulmonary hypertension. The presence of the anti-U1RNP autoantibodies in the sera of patients is characteristic for MCTD (1,2). Vasculopathy with proliferative vascular lesions in the intima of muscular arteries and arterioles and vast mononuclear cell infiltration in different tissues have been described in MCTD (3-5). The abundant production of endothelin-1, as well as endothelial cell proliferation in the vessel walls suggest that endothelial damage may be crucial for the pathogenesis of MCTD (3-6).

Anti-endothelial cell antibodies (AECA) have been detected in many rheumatic disorders including systemic lupus erythematosus (SLE), systemic sclerosis (SSc), polymyositis/dermatomyositis (PM/DM), glomerulonephritis, most forms of systemic vasculitis, rheumatoid arthritis (RA) with or without vasculitis, thrombotic thrombocytopenic purpura and hemolytic uraemic syndrome (TTP/HUS), as well as several other disorders (7-13; reviewed in 14, 15). Immune-mediated/inflammatory damage of the vessel wall may be involved in the pathogenesis of all these conditions. AECA may be directed against several endothelial antigens including DNA, ribosomal proteins, extracellular matrix molecules and other glycoproteins (reviewed in 14, 15). AECAmay up-regulate endothelial adhesion molecule expression in systemic vasculitides (15). AECA have been detected by us and others in MCTD (6, 15-17). However, less information is available on the possible pathogenic effects of AECA in active versus inactive MCTD. In addition, the effects of MCTD-associated AECA on endothelial adhesion molecule expression is also unknown.

In this study, we assessed sera of MCTD patients for AECA concentrations and compared that to healthy subjects. AECA production in active versus inactive MCTD patients was compared. In addition, we searched for possible associations between serum AECA concentrations and clinical manifestations of MCTD. Finally, the effects of $AECA^{high}$ versus $AECA^{low}$ sera on endothelial adhesion molecule expression were also investigated.

Materials and methods

Patients and serum samples

Thirty-three patients were included in the study. The diagnosis of MCTD was established according to the criteria by Alarcon-Segovia and Villareal (2). Clinically active disease was defined as described by Burdt *et al.* (9). Briefly, patients were considered to be inactive, when there was no evidence of major organ involvement except mild Raynaud's phenomenon, they had normal overall functional capacity for more than one year on a therapeutic regimen of <20 prednisone per day. Active patients had severe disease involving a number of organs and required aggressive therapy with repeatedly administered > 30 mg prednisone per day (Table I).

The age of these 33 MCTD patients ranged between 21-59 years (mean age: 44 years). The male:female ratio was 2:31. Sera were obtained from all patients, as well as from 30 healthy volunteers (age 20-55 years, mean age: 38 years, male:female ratio: 1:4). All serum samples were stored in 2 ml aliquots at –20ºC until use.

Determination of anti-U1RNP autoantibodies in sera

Autoantibodies to U1RNP were determined by ELISA(Cogent Diagnostics). The normal range for this assay was ≤ 18 μg/ml. All patients' sera were positive for anti U1RNP, which is elementary for the diagnosis of MCTD (range: 21-46 mg/ml). In addition, no antiphospholipid antibodies could be detected in the sera of any patient excluding the possibility of the interference of these antibodies with AECA (data not shown).

Human umbilical vein endothelial cell (HUVEC) culture

Endothelial cells were isolated from hu-

Table I. Clinical characteristics of patients with mixed connective tissue disease.

Pt.	Age	Sex	Clincal presentation	Activity	Treatment
1	51	F	A,R,M,E,PH	Yes	CS, Cph.,Dic.
2	43	F	A,R,Sw, M,E,	Yes	CS, Ca, Pf.
3	35	F	A,R,CNS,E	Yes	CS, Ca, Cph., Pf.
4	47	F	A,R,Sw,M,E,Scl	Yes	CS, Ca, Pf.
5	38	F	A,M,E, P,ILD	Yes	CS, Cph.
6	40	F	A,R,E,Scl	Yes	CS, Cph. Pf.
7	38	F	R,E,P,V,Scl	Yes	CS, Cph.
8	53	M	A,E,P,V,Scl	Yes	CS, Chl.
9	55	F	A,R,P,Scl	Yes	CS, Cph., Pf.
10	34	F	R,P,E,M,	Yes	CS, Ca, Pf.
11	43	F	A,R,Sw,PH	Yes	CS, Cph.,Pf.
12	48	F	A,E,S,PH	Yes	CS, Cph., Dic.
13	29	F	R,M,Scl, V	Yes	CS, Chl.
14	43	F	A,R,Sw,PH,ILD	Yes	CS, Cph., Pf.
15	36	M	A,Scl,ILD,E,	Yes	CS, Cph.
16	42	F	A,R,E,M, Sw,PH	Yes	CS, Cph.,Dic., Prost.
17	35	F	A,R,Sw, CNS,PH,	Yes	CS, Cph., Dic.,Prost.
18	43	F	A,Scl,ILD,E,	Yes	CS, Cph.,Pf.
19	38	F	A,R,Scl,P,PH	Yes	CS, Cph., Dic.
20	57	F	A,R,Sw,P	No	CS, NSAID, Pf.
21	48	F	A,R,Scl,E,	No	NSAID, Pf.
22	45	F	R,Sw,E,ILD,P	No	NSAID, Ca, Ace
23	54	F	A,R,Scl,E	No	CS, Ca, Pf.
24	56	F	A,R,L,ILD,M	No	CS, Ca, Ace, Pf.
25	45	F	A,E,V,L	No	CS, Chl.
26	47	F	A,R,CNS,E,M	No	CS, Ace, Pf.
27	53	F	A,R,Sw,Scl,P	No	NSAID
28	21	F	A,P,E,ILD	No	CS, Ace, Pf.
29	39	F	A,R,V,Sw	No	Chl., Ca
30	54	F	A,R,P,Scl	No	NSAID, Pf.
31	59	F	A,E,Sw,P	No	CS, Ca,
32	45	F	A,R,Sw,M,CNS	No	CS, Ca, Ace, Pf.
33	58	F	A,R,Scl,E,ILD,V	No	CS, Ca, Ace, Pf.

Sex: F: female; M: male. *Clinical:* A: arthritis; CNS: central nervous system; E: esophageal hypomotility; ILD: interstitial lung disease; M: myositis; P: pericarditis or pleuritis; PH: pulmonary hypertension; R: Raynaud's phenomenon; Scl: sclerodactily; Sw: swollen hands; V: vasculitis.
Treatments: Ace: angiotensin convertase enzyme inhibitor; Ca: Calcium channel blocker; Chl: chloroquine; Cph: cyclophosphamide; CS: cortocosteroids; Dic: dicoumarol; NSAID: non-steroidal anti-inflammatory drugs; Pf: pentoxyfilline; Prost: prostacyclin.

man umbilical cord veins. Briefly, human umbilical cords were obtained from the Department of Obstetrics at our university. Veins were filled with collagenase solution (0.1%, Worthington). Harvested endothelial cells were routinely cultured in MEM (Gibco-BRL) supplemented with 40% pooled, heat-inactivated (56ºC, 30 minutes) human serum and antibiotics. Two days before the experiments, endothelial cells were gently detached with a 0.1% trypsin/0.02% EDTA (Sigma) (1:1) solution. Cells were seeded on gelatin-coated plastic wells (24-well plates, Costar) at a density corresponding to 100,000 cells/cm^2 in MEM containing only 30% human serum and antibiotics. Prior to the assay, cells were identified as ECs by immunofluorescent staining with rabbit anti-human factor VIII related antigen antibody (Behring).

AECA ELISA

After the second passage, HUVEC were seeded at 2 x 10^4/100 µl on sterile polystyrene 96-well plates (Costar). Cells were cultured for 24 hours then the plates were washed 3 times with phosphate buffered saline (PBS, Sigma), pH 7.4 and fixed with 0.2 glutaraldehyde for 15 minutes at 22ºC, then washed twice with PBS containing 0.2% bovine serum albumin (BSA, Sigma). After 3 washes, 100 µl serum, diluted 1: 20 in PBS was added to each well in triplicate and incubated for 2 hours at room temperature. After three washes, 100 µl of peroxidase-conjugated goat anti-human IgG F(ab')$_2$ antibody and peroxidase-conjugated goat anti-human IgM antibody (Cappel Laboratories) diluted 1:500 in PBS containing 2% BSAwere added to each well for an additional 2 hours. Non-specific immunoglobulin binding was prevented by blocking steps using goat serum. Then 100 µl of 4 mmol/l *O*-phenylene diamine dihydrochloride (Sigma) and 1.3 mmol/l hydrogen peroxyde (Sigma) in 0.1 mmol/l citrate buffer pH 5.0, were added to each well for 20 minutes. The reaction was terminated by the addition of 100 µl of 2 mmol/l sulfuric acid, and the optical density was measured at 492 nm by an ELISAmicroplate reader.

Two positive and two negative control sera were included on each plate. Samples were considered AECA^{high} if OD values of patients'samples were greater than the mean + 2SD value detected in sera of healthy subjects. The intra-assay and inter-assay coefficients of variation (CV) were 5% and 9% respectively.

Five AECA^{high} and AECA^{low} sera from MCTD patients and sera from 5 healthy subjects were pooled and IgG was precipitated by ammonium sulfate (Sigma) and further purified with the use of protein A-Sepharose colums. The IgG fractions were eluted with 100 mM glycine-HCl buffer pH 3.0 and immediately dialysed extensively against PBS. The dialysed preparations were sterilized by filtration through a nitrocellulose filter (0.22 µm; Millipore). IgG preparations were tested in the AECA assay. All preparations were tested for endotoxin content using Limulus assay and no contamination was observed.

In order to prevent binding of antinuclear antibodies present in the sera, control preliminary experiments using unfixed cells were also performed, and no differences were found between the use of fixed versus unfixed cells (data not shown).

Adhesion molecule expression on HUVEC

HUVEC were cultured for 24 hours on sterile polystyrene 96-well plates (Costar) at 2 x 10^4/100 ml then cells were washed with MEM. After removing the medium, ECs were incubated for 4 hours at 37ºC in a humified 5% CO_2 atmosphere with purified IgG from AECA^{high} and AECA^{low} patients, as well as controls. All experimental sets included 5 ng/ml recombinant human interleukin (IL)-1β (R and D Systems) as positive control, as this cytokine is known to up-regulate endothelial adhesion molecule expression.

After further incubation, HUVEC monolayers were fixed with cold methanol for 5 min at 4ºC, incubated with PBS containing 10% FCS to reduce non-specific binding, and incubated for one hour at 37ºC with 100 µl murine monoclonal antibody against E-selectin (250 ng/ml) (Becton Dickinson), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (both R&D Systems) in PBS containing 10% goat serum. HUVEC were then washed 3 times with PBS and 100 µl peroxidase-conjugated goat anti-mouse IgG secondary antibody (250 ng/ml; Becton Dickinson) in PBS containing 20% goat serum were added to each well, followed by incubation at 37ºC for 60 min. The wells were washed with 100 µl of 4 mmol/l *O*-phenylene diamine dihydrochloride (Sigma) and 1.3 mmol/l hydrogen peroxyde (Sigma) in 0.1 mmol/l citrate buffer pH 5.0, were added to each well for 20 minutes.

The reaction was terminated by the addition of 100 µl of 2 mmol/l sulfuric acid, and the optical density was measured at 492 nm by a Beckman ELISA microplate reader.

Statistical analysis

The mean values of data for different groups of patients and controls, as well as the standard deviations (SD) of the mean were calculated. Results were compared utilizing the Student's t-test for unpaired data and χ^2 test. Pvalues < 0.05 were considered to be statistically significant

Results

Clinical manifestations of MCTD patients

Altogether 33 patients with MCTD and 30 normal controls were involved in the study. Summary of the clinical symptoms of the patients are shown in Table I. Based on the criteria described above, 19 patients had active (patient nos. 1-19) and 14 (nos. 20-33) had inactive disease. Regarding vascular manifestations, 7/19 active patients had pulmonary hypertension, while none of inactive patients had this manifestation. Raynaud's phenomenon was seen in 13/19 active and 11/14 inactive patients. 3/19 active and 3/14 inactive patients had localized vasculitis.

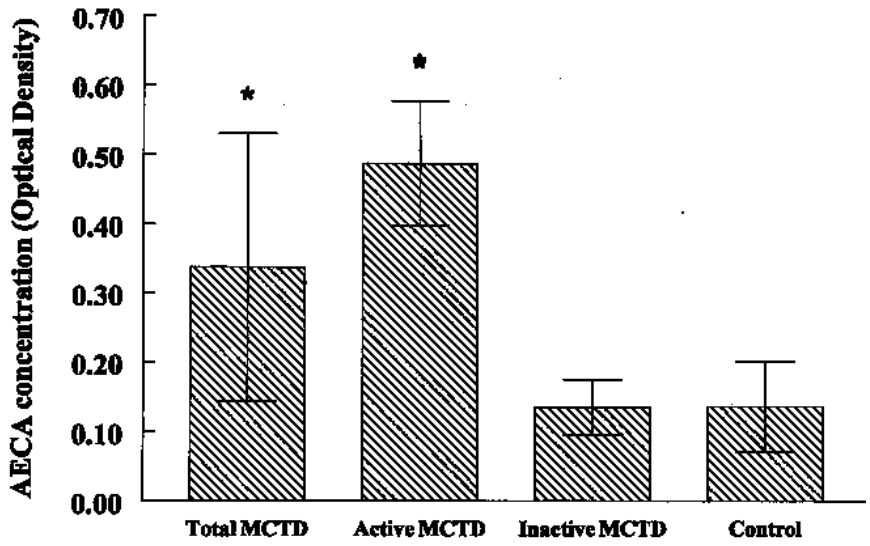

Fig. 1. AECAlevels in the sera of MCTD patients, patients with active and inactive disease, as well as normal controls as determined by optical density measurements. Asterisks indicate significant differences in comparison to inactive MCTD and controls ($p < 0.05$).

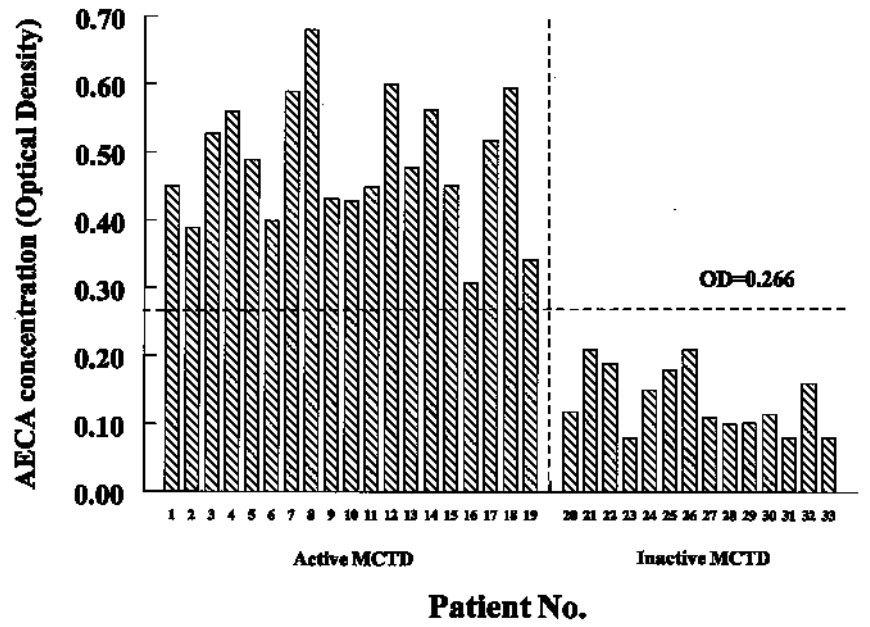

Fig. 2. AECAlevels in the sera of 33 MCTD patients, including patients with active and inactive disease as determined by optical density measurements. The horizontal dashed line indicates the mean + 2SD OD value of control subjects (OD = 0.266), as a threshold between AECA^{high} and AECA^{low} samples. The vertical dashed line separates patients with active (n = 19) and inactive MCTD (n = 14).

AECA is the sera of MCTD patients and controls

Determination of AECA in the sera from the 30 healthy volunteers resulted in a mean OD of 0.136 ± 0.065. We observed significantly increased production of AECA in MCTD patients (OD = 0.337 ± 0.193) compared to controls ($p < 0.05$). Patients with active MCTD had significantly higher amounts of AECA in their sera (OD = 0.487 ± 0.090) than did patients with inactive disease (OD = 0.135 ± 0.040) ($p < 0.05$). There was no difference in terms of serum AECA concentrations between inactive patients and controls (Fig. 1).

As serum samples of MCTD patients were then considered as $AECA^{high}$ if OD was higher than the mean + 2SD of controls, the threshold between $AECA^{high}$ and $AECA^{low}$ was OD = 0.266. Interestingly, sera from all 19 patients with active disease were $AECA^{high}$ (OD range: 0.308 - 0.680), while sera from all 14 inactive MCTD patients were $AECA^{low}$ (OD range: 0.080 - 0.210) (Fig. 2).

Possible correlations between serum AECA levels and various organ manifestations

The involvement of various organs of our MCTD patients in seen in Table I. Pulmonary hypertension was detected in 7 patients, all with active disease. These patients exerted a tendency of increased serum AECA levels (OD = 0.452 ± 0.080) compared to patients lacking this manifestation (OD = 0.307 ± 0.039) ($p = 0.07$) (Fig. 3). We did not find any correlations between other types of organ involvement and serum AECA concentrations (data not shown).

Effect of MCTD IgG fractions on endothelial E selectin expression

HUVEC monolayers were pretreated with purified IgG obtained from 5-5 $AECA^{high}$ and $AECA^{low}$ MCTD sera, as well as control sera. The lowest concentration of IgG producing significantly up-regulated endothelial adhesion molecule expression was 200 μg/ml (data not shown).

After 4 hours of incubation, IgG purified from the 10 MCTD sera up-regulated E-selectin expression on HUVEC (OD = 0.415 ± 0.290) compared to IgG from control sera (OD = 0.131 ± 0.081), although this difference was not statistically significant ($p = 0.055$) (Fig. 4). However, the stimulatory effect of $AECA^{high}$ MCTD sera on endothelial E-selectin expression (OD = 0.651 ± 0.190) was significantly increased in comparison to IgG purified from $AECA^{low}$ MCTD sera (OD = 0.178 ± 0.109) or control sera (OD = 0.131 ± 0.081) ($p < 0.05$) (Fig. 4). The effect of IgG from $AECA^{high}$ sera on endothelial E-selectin expression (OD = 0.651 ± 0.190) did not differ significantly from that of the positive control IL-1β (OD = 0.906 ± 0.110). In addition, there was no significant difference between the effects of IgG from $AECA^{low}$ MCTD sera and control sera (Fig. 4).

We also tested IgG purified from AEC-

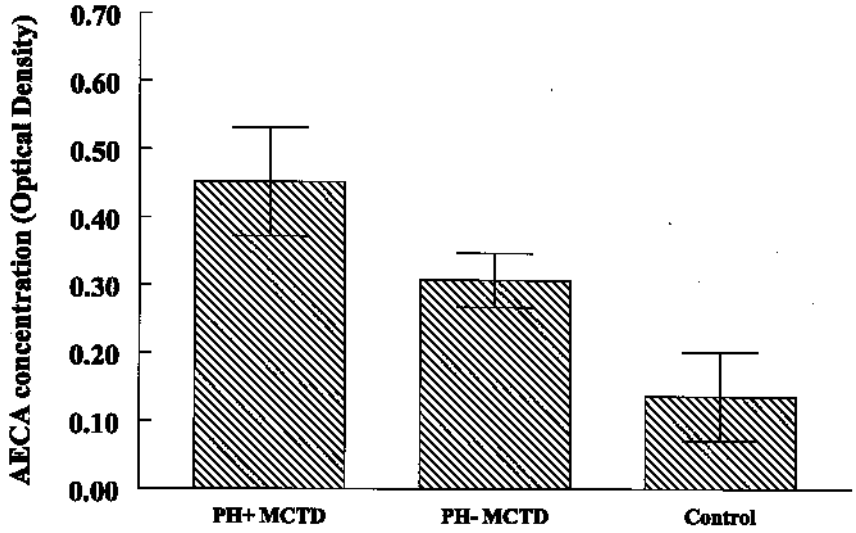

Fig. 3. Association between serum AECA levels and pulmonary hypertension in MCTD patients as determined by optical density measurements. Asterisks indicate significant differences in comparison to controls ($p < 0.05$). No statistically significant difference was found between patients with and without pulmonary hypertension. PH: pulmonary hypertension.

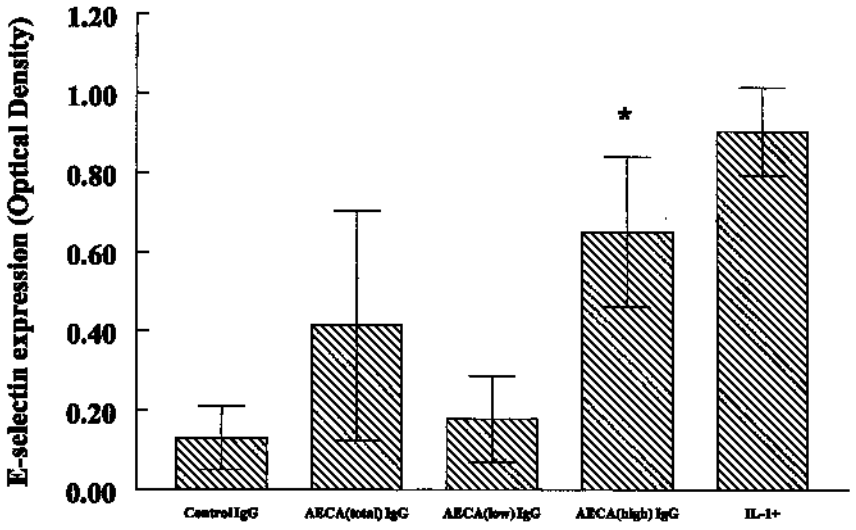

Fig. 4. E-selectin expression on cultured HUVEC induced by IgG purified from the sera of all MCTD patients ($AECA^{total}$), $AECA^{high}$ and $AECA^{low}$ sera and control sera as determined by optical density measurements. IL-1-induced endothelial E-selectin expression was used as positive control. The asterisk indicate significant differences in comparison to AECAlow IgG and control IgG ($p < 0.05$).

A^{high} and $AECA^{low}$ $AECA^{low}$ MCTD sera on endothelial ICAM-1 and VCAM-1 expression. However, no up-regulation of these adhesion molecules was found in comparison to IgG from control sera (data not shown).

Discussion

Clinical and pathological investigations performed in MCTD suggest that deterioration of the vasculature may determine the disease course. Obliterative vasculopathy associated with endothelial cell proliferation are characteristic complications of MCTD, especially in small pulmonary arteries and arterioles. Pulmonary hypertension is a major cause of mortality of MCTD patients (2-4).

AECA is a heterogeneous group of antibodies, directed against phospholipids and proteins located on the cell membrane of endothelial cells (12,18, 19). The exact binding mechanism and the pathogenic role of AECA on endothelial cells is somewhat unclear (20, 21). Several possible target antigens for AECA have been identified on endothelial cells including DNA, ribosomal proteins, extracellular matrix components including heparan sulfate, fibronectin and vimentin, β_2 glycoprotein I, proteinase 3 and myeloperoxidase (ANCA), the CD36 antigen and others (reviewed in 15). It is possible that the target structures are different in various pathological conditions.

AECAhave been detected in almost all forms of systemic vasculitis, systemic autoimmune diseases including SLE, SSc, RA with or without vasculitis, PM/DM, MCTD and antiphospholipid syndrome, transplantation, as well as various other disorders (reviewed in 14, 15). AECA in sera of patients with SLE are capable to initiate complement binding and to disrupt the cultured endothelial cell monolayer (22). In a small proportion of SLE and SSc patients AECAdamage cultured endothelial cells in the presence of mononuclear cells by antibody-dependent cellular cytotoxicity (23,24). AECA levels were correlated with clinical activity in patients with lupus nephritis and IgA nephropathy (25,26). In SLE and systemic vasculitides, AECAincreased IL-1, IL-6 and IL-8 secretion by endothelial cells (27). In Kawasaki disease, AECA of IgG and IgM isotypes destroyed cytokine-treated endothelial cells but had no effect on resting cells (28). In TTP/HUS, the patients' sera caused lysis of endothelial cells (9).

We and others have detected AECA in MCTD patients (6, 15-17). AECA-containing MCTD sera damaged the endothelial cells by rabbit complement. Using ELISA we showed AECA binding to endothelial cells. In addition, AECA concentrations showed a close association with elevated plasma endothelin-1 levels in MCTD (6, 17). However, little information is available on the possible endothelial target structures and the associations between serum AECAlevels and clinical activity in MCTD. In the present study, significantly increased AECAproduction was observed in 33 MCTD patients compared to 12 controls, as well as between patients with active disease compared to inactive MCTD patients or controls. When dividing MCTD sera to $AECA^{high}$ and $AECA^{low}$ based on the AECA production of healthy subjects, all patients with active disease had $AECA^{high}$, while all inactive MCTD patients had $AECA^{low}$ sera.

Regarding possible associations between AECA production and organ manifestations in MCTD, in one study, others found correlation between high levels of AECA directed towards cytokine-treated endothelial cells and the presence of pulmonary fibrosis in these patients (16). Here we found a non-significant association between serum AECA levels and pulmonary hypertension in our MCTD patients. Regarding other systemic autoimmune diseases, in SSc, an association between AECA production and pulmonary hypertension, digital ulcers, capillaroscopic abnormalities and alveolar-capillary dysfunction was observed (29). In RA, AECAare found more frequently in patients with associated vasculitis than without vascular involvement (30). In SLE, AECAwere correlated with nephritis and vasculitis (24).

In our hands, IgG isolated from the sera of MCTD patients exerted a tendency to up-regulate E-selectin, but not ICAM-1 and VCAM-1 expression on cultured HUVEC. IgG purified from $AECA^{high}$ MCTD sera had significantly increased stimulatory effect on endothelial E-selectin expression than did $AECA^{low}$ MCTD sera or control sera. E-selectin expression was detected on endothelium of cutaneous dermal vessels in patients with MCTD (31). In SLE, endothelial binding of antiphospholipid antibodies has been shown to up-regulate E-selectin expression on cultured endothelial cells (32). HUVEC incubated with AECA IgG from Wegener's granulomatosis patients resulted in the up-regulation of endothelial E-selectin expression (33). AECA IgG from SLE and vasculitis patients, as well as interactions between endothelial β_2 glycoprotein I and anti-β_2 glycoprotein I antibodies result in increased intercellular adhesion (21, 27).

In summary, there is an increased production of AECA in MCTD patients compared to controls. Serum AECA levels may be associated with clinical activity. One possible mechanism of AECA in MCTD is the up-regulation of endothelial E-selectin thus leading to increased adhesion of inflammatory cells to the endothelium. Therefore, AECA may have important effects in the pathogenesis of MCTD and the assessment of serum AECAlevels may be a useful marker of endothelial activation and clinical activity in this disease.

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