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Behçet's Disease Sera Containing Antiendothelial Cell Antibodies Promote Adhesion of T Lymphocytes to Cultured Human Dermal Microvascular Endothelial Cells

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Abstract

Antiendothelial cell antibodies (AECA) have been detected in the sera of patients of autoimmune diseases showing vasculitis. Using IgM-ELISA, we found AECA in 42 (56%) of 75 sera samples from patients with Behçet's disease in a previous study. All of the 15 AECA-positive sera of Behçet's disease patients had an increased expression of the intercellular cell adhesion molecule-1 (ICAM-1), 93.3% of the sera induced the vascular cell adhesion molecule-1 (VCAM-1), and 100% of the serum induced the E-selectin molecule on human dermal microvascular endothelial cells (HDMEC). After stimulation of HDMEC with AECA-positive sera of Behçet's disease patients, the expression of ICAM-1 and VCAM-1 on HDMEC increased significantly at 4 hours, reaching a peak at 16 hours. Expression of E-selectin was induced at 1 hour after stimulation with a peak at 4 hours and it decreased thereafter. Adherence of T lymphocytes to HDMEC increased significantly after stimulation with AECA-positive sera from Behçet's disease patients. Also, the adherence of T lymphocytes to HDMEC increased at 4 hours and returned to its normal level at 48 hours. These results show that AECA-positive sera of Behçet's disease patients are capable of activating HDMEC to promote the adherence of T lymphocytes to increase the expression of ICAM-1, VCAM-1, and E-selectin on the cell surfaces. The whole process may play an important role in the pathogenesis of vasculitis in Behçet's disease.

Key Words: Antiendothelial cell antibodies, Behçet's disease, ICAM-1, VCAM-1, E-selectin, T lymphocyte

INTRODUCTION

Behçets disease (BD) is a chronic relapsing vasculitis affecting multiple organ systems. There is a higher incidence of BD in East Asian and Mediterranean countries. Serious complications such as blindness or intestinal perforations may occur. The pathogenesis of BD remains obscure and there are no definitive diagnostic tests to aid in the diagnosis except for clinical symptoms. Vascular endothelial injury seems to be responsible since clinically recurrent thrombophlebitis, thrombosis, erythema nodosum and cutaneous vasculitis occur frequently.[1] Histopathologic changes consisting of perivascular monoclonal cellular infiltrate, endothelial cell swelling or necrosis, partial luminal obliteration and occasional fibrinoid necrosis of the vessels have been observed.[2]

The epitopes on the surface of endothelial cells (EC) are immunologic targets of cellular cytotoxicity in autoimmune diseases such as systemic lupus erythematosus (SLE), systemic sclerosis (SS), Kawasakis disease (KS) and rheumatoid arthritis (RA).[3] Antiendothelial cell antibodies (AECA) have been demonstrated in sera from patients with a variety of vascular disorders including SLE, SS, RA, graft-versus-host disease, thrombocytopenic purpura, multiple sclerosis, and autoimmune hypoparathyroidism.[4-7] AECA may play an important role in vascular injury in systemic vasculitis. It has been suggested that when AECA attach to EC, they can induce damage to EC either through complement action, cytotoxic T cells, natural killer cells or by modifying the function of EC.

Previous studies using human umbilical vein endothelial cells (HUVEC) have found 18 to 33% prevalence of AECA in BD.[8,9] We have also previously demonstrated that circulating AECA were present in the sera of BD patients.[10] However, it remains

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unclear whether AECA play a direct role in causing endothelial dysfunction in BD or merely represent clinical markers of disease activity or progression.

Adherence of leukocytes to vascular EC is regulated by the treatment of biological response modifiers (BRM) to EC.[11,12] The binding of leukocytes to EC is governed by the expression of cell adhesion molecules which are regulated by BRM.[13] T lymphocyte infiltration across the vessel wall is prominent in BD lesions.[14]

In this study, we used enzyme-linked immunosorbent assay (ELISA) and immunofluorescence flow cytometric analysis, which allowed detection of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin on human dermal microvascular endothelial cells (HDMEC) following their incubation with AECA-positive sera of BD patients. In addition, we used an in vitro model of the vascular wall consisting of HDMEC monolayers to study the binding of T lymphocytes to HDMEC after stimulating with AECA-positive sera of BD patients.

MATERIALS AND METHODS

Subjects

Sera were obtained from healthy normal volunteers (n=60), from patients with BD (n=75), each of whom fulfilled the diagnostic criteria of the International study group for BD.[15] All serum specimens were obtained at a time when patients had active disease. Sera were collected and stored at $-70^{\circ}C$ until use.

Cell culture

HDMEC were isolated from human neonatal foreskins by trypsinization and percoll gradient centrifugation as described previously.[16,17] Cells were cultured in endothelial basal media (Clonetics Corp., San Diego, CA, U.S.A.) with epidermal growth factor 5 ng/ml (Clonetics), hydrocortisone acetate 1 mg/ml (Sigma Chemical Co., St. Louis, MD, U.S.A.), dibutyryl cyclic AMP 5×10^{-5}M (Sigma), penicillin 100 U/ml, streptomycin 100 mg/ml, amphotericin B 250 mg/ml (Sigma), and 30% human serum (Irvine Scientific, Santa Ana, CA). The resulting cell cultures were free of contaminating fibroblasts as assessed by morphologic and immunologic criteria. Experiments were conducted with EC at passages 2-6.

ELISA for AECA

The sera were screened for the presence of AECA using ELISA. HDMEC were plated in 96-well plates and allowed to grow to confluence over 24 h. A total of 100 μl of sera diluted 1 : 200 in Hanks balanced salt solution (HBSS) with divalent cations (Irvine) and 1% bovine serum albumin (Sigma) was added to each well and the plates were incubated at $37^{\circ}C$ for 1h. After washing, 100 μl of peroxidase-conjugated goat anti-human IgG (Sigma) or IgM (Sigma) diluted 1 : 1,000 by HBSS with divalent cations and 5% newborn calf serum (Gibco), was added to each well and plates were incubated for 1 h. The plates were again washed and the binding of antibody was quantitated colorimetrically by the addition of tetramethylbenzidine (TMB, 1 mg/ml, Sigma). One ml of a 100 mg/ml stock solution of TMB in acetone was added to 100 ml of distilled water. Ten microliters of 30% H_2O_2 was added immediately prior to use. The chromogenic reaction was stopped with 25 μl 8 N H_2SO_4 and the plates were read spectrophotometrically at 450 nm on an ELISA reader (Dynatech Laboratories Inc., Alexandria, VA, U.S.A.). All data points were done in triplicate.

Sera samples from negative healthy controls, AECA-negative BD patients, AECA-positive BD patients, and sera samples depleted AECA from sera of AECA-positive BD patients using gel chromatography were used in ELISA and flow cytometry for the expression of adhesion molecules.

ELISA for detection of adhesion molecule expression

HDMEC were plated in 96-well flat-bottomed microtiter plates and allowed to grow to confluence over 24 h at a concentration of 4×10^{4} cells per well. HDMEC were incubated with AECA-positive sera of BD patients. A total of 100 μl of either Anti-ICAM-1 antibody (84H10, Immunotech Inc., Westbrook, ME, U.S.A.), anti-VCAM-1 antibody (51-10C9, Pharmingen, San Diego, CA, U.S.A.), or anti-E-selectin antibody (1.2B6, Immunotech Inc., Westbrook, ME, U.S.A.) was added to each well and the plates were incubated at $37^{\circ}C$ for 1 h. After washing, 100 μl of peroxidase-conjugated goat anti-mouse IgG (Sigma), diluted 1 : 500, was added to each well and the plates were incubated for 1 h. The plates

were again washed and the binding of antibody was quantitated colorimetrically by the addition of tetramethylbenzidene (TMB, 1 mg/ml, Sigma). One ml of a 100 mg/ml stock solution of TMB in acetone was added to 100 ml of distilled water. Ten microliters of 30% H_2O_2 were added immediately prior to use. The chromogenic reaction was stopped with 25 μl 8 N H_2SO_4 and the plates were read spectrophotometrically at 450 nm on an ELISA reader.

Flow cytometric analysis

HDMEC were plated in 96-well flat-bottomed microtiter plates and allowed to grow to confluence over 24 h at a concentration of 4×10^4 cells per well. HDMEC were incubated with AECA-positive sera of BD patients. HDMEC were removed by incubation with 1% bovine serum albumin (Gibco) and 3 mM ethylenediamine tetracetic acid. HDMEC were then washed in Hanks balanced salt solution (Gibco), counted and aliquotted for staining. Following incubation, HDMEC were incubated with either anti-ICAM-1 antibody, anti-VCAM-1 antibody or anti-E-selectin antibody for 30 min on ice, washed and then incubated with FITC-conjugated anti-mouse IgG (Sigma) for 30 min. After another 30 min incubation, HDMEC were washed and resuspended in phosphate buffered saline (pH 7.2) with 0.5% bovine serum albumin. Propidium iodide was added immediately before flow cytometric analysis to identify dead cells. Fluorescence was examined on a fluorescence activating cell sorter (FACStar, Becton-Dickinson, Lincoln, NJ, U.S.A.).

Separation of peripheral blood mononuclear cells and T lymphocytes

Peripheral blood monocytes from healthy normal donors were separated using Ficoll-Hypaque (Organon Technik Corp., Durham, NC, U.S.A.) density gradient centrifugation. Adherent mononuclear cells were separated from nonadherent mononuclear cells by adherence to plastic at 37°C for 1 h. To eliminate cells other than T lymphocytes, selective killing was performed with low-tox rabbit complement (Cedarlane Lab., Westburg, NY, U.S.A.) and monoclonal antibodies CD19 (Leu 12, Becton-Dickinson, San Jose, CA, U.S.A.), CD11c (Leu-M5, Becton-Dickinson), and-Mo-1 (Mac-1, Coulter Immunology, Hialeah, FL, U.S.A.), anti-HLA-DR (Becton-Dickinson), and anti-HLA-DR (Serotec, Oxford, UK). The cocktail of monoclonal antibodies was added to the cells and incubated at room temperature for 30 min. After the addition of T-cell medium consisting of RPMI 1640 (Gibco), 2 mM L-glutamine, 10% fetal bovine serum (Gibco), 50 mM 2-mercaptoethanol (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin, the pellets were resuspended in the complete T-cell media.

T lymphocyte-HDMEC adherence assay

HDMEC were plated in 96-well flat bottomed microtiter plates and allowed to grow to confluence over 24 h at a concentration of 4×10^4 cell per well. HDMEC were preincubated with either culture media alone or by incubating with AECA-positive sera of BD patients. T lymphocytes were labeled with ^{51}Cr (Dupont, Boston, MA, U.S.A.) by incubating 100 μCi per 10^6 cells for 16 h at 37°C. They were washed, suspended to 4×10^6/ml in RPMI with 10% fetal bovine serum and then 100 ml of cell suspension was added to each well containing HDMEC and incubated for 1h. After incubation at 37°C, the plates were washed twice, 100 μl of 1% Triton-X (Sigma) were added to each well, and the contents of each well were harvested and counted in a gamma counter. The percentage of bound T lymphocytes was calculated as follows: Percent of T lymphocytes binding = (adherent counts - background counts)/(total counts added per well - background counts) × 100

Statistics

Mean values and standard deviation (SD) were calculated in all experiments. Data were analyzed using multiple comparison (Tukey's Studentized Range) test after one-way ANOVA analysis. P values less than 0.05 were considered significant.

RESULTS

IgM antibody reactivity to cultured HDMEC

Fig. 1 demonstrates the mean optical density (OD) of sera from 60 normal controls of 0.111 ± 0.035. When the values were higher than the mean OD for the normal controls plus three times the standard deviation, the OD was considered reactive. 42 (56%) 1of 75 sera from patients with BD had high titers of IgM AECA with an OD value of 0.457 ± 0.065

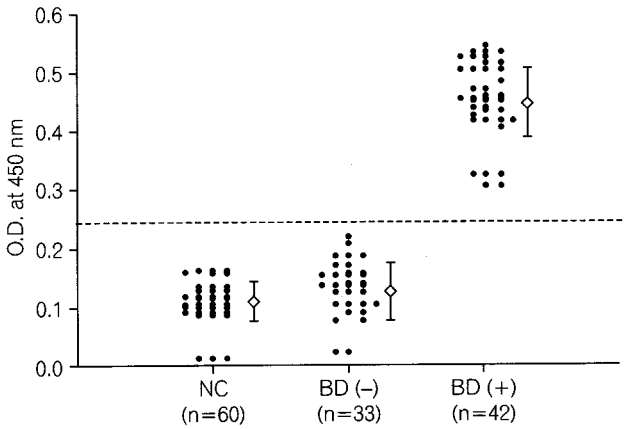

Fig. 1. IgM antibody activity to cultured HDMEC in serum of patients with BD. HDMEC monolayer was incubated with sera from normal control (NC), AECA-negative sera of BD patients {BD (−)}, and AECA-positive sera of BD patients {BD (+)}. The IgM antibody activity was measured with ELISA and each dot is the mean result of an individual serum performed in triplicate. The mean ±SD for each group is shown. The horizontal dotted line represents the level of the mean OD plus three times the SD for normal controls.

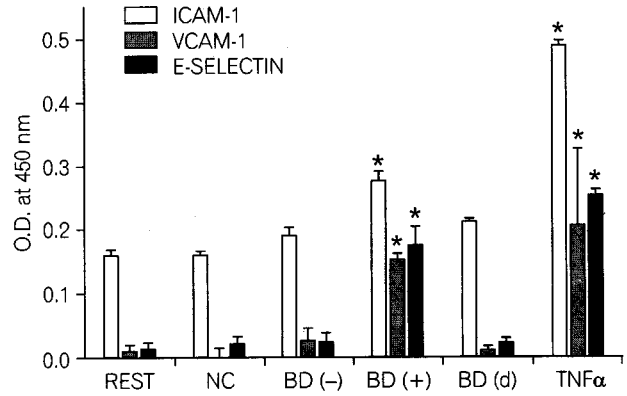

*Fig. 2. Expression of ICAM-1, VCAM-1 and E-selectin molecule on HDMEC after incubation with sera of BD patients. After 16 hours of incubation with normal control sera (NC), AECA-negative sera of BD patients {BD (−)}, AECA-positive sera of BD patients {BD (+)}, IgM-depleted sera of AECA-positive sera of BD patients {BD (d)}, and TNFa, the adhesion molecule expression was investigated by using monoclonal antibody and read spectrophotometrically at 450 nm on an ELISA reader. *p<0.05 vs. untreated HDMEC.*

Table 1. The Effects Of Behçet's Disease Serum in the Expression Changes of Adhesion Molecules on HDMEC

Group	No. sera	Adhesion molecules		
		ICAM-1 (%)	VCAM-1 (%)	E-selectin (%)
Normal control	15	0* (0)	0 (0)	0 (0)
BD (−)	15	4 (26.7)	0 (0)	2 (13.3)
BD (+)	15	15 (100)	14 (93.3)	15 (100)

*No. of sera showing expression changes of endothelial cell adhesion molecules after the treatment of sera of normal healthy controls or Behçet's disease sera.
BD (−): antiendothelial cell antibody negative Behçet's disease sera-treated group.
BD (+): antiendothelial cell antibody positive Behçet's disease sera-treated group.

and 33 (44%) were non-reactive of the IgM-AECA with an OD value of 0.134±0.046. There were no significant differences of the mean duration of disease, sex ratio, and age distribution between the AECA-positive and AECA-negative groups. However, 16.7% of the AECA-positive group had thrombophlebitis compared to none of the AECA-negative group (data not shown).

Expression of adhesion molecules on HDMEC after stimulation with AECA-positive sera of BD patients

Results of ELISA showed the expression changes of ICAM-1, VCAM-1, and E-selectin on HDMEC in response to AECA-positive sera. All of the 15 AECA-positive patients with BD had an increased expression of ICAM-1 and 93.3% of the serum induced the expression of VCAM-1 and 100% of the serum induced the E-selectin molecules on HDMEC (Table 1). The culture of HDMEC with unstimulated control and AECA-negative sera failed to express the ICAM-1, VCAM-1, and E-selectin molecules. IgM AECA-depleted sera of AECA-positive sera of BD patients did not show any increase in the expression of endothelial adhesion molecules. However, stimulation of HDMEC with TNFa led to an increase in endothelial adhesion molecule expression (Fig. 2).

Flow cytometric analysis of HDMEC incubated with IgM-positive AECA sera of BD patients demonstrated an increase in the expression of ICAM-1, VCAM-1 and E-selectin molecules (Fig. 3). Time course experiments identified the duration of incubation required to achieve maximal expression of adhesion molecules on HDMEC. Levels of expression of ICAM-1 and VCAM-1 molecules increased at 1 h, peaked at 16 h and then returned to basal levels at 24 h ($p<0.05$). In contrast, the expression of E-

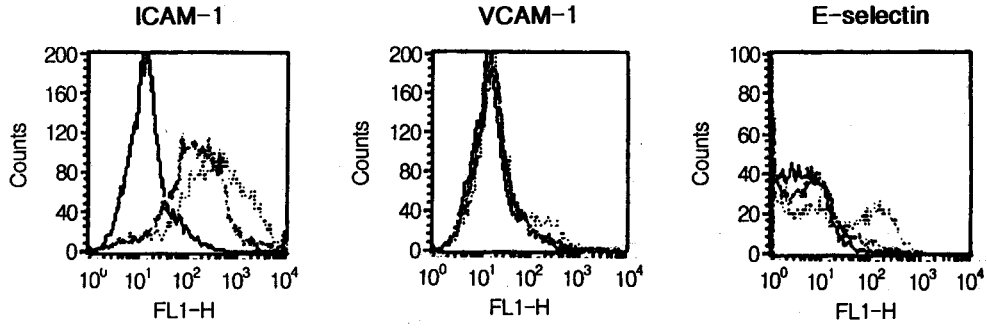

Fig. 3. Flow-cytometric analysis of the expression of endothelial cell adhesion molecules after stimulating HDMEC with AECA-positive sera of BD patients was analyzed by FACStar. Irrelevant antibody (black line) incubated HDMEC and unstimulated HDMEC (gray line) do not express EC adhesion molecules. After 16 hours of incubation of AECA-positive sera of BD patients (dotted line), the expression of endothelial cell adhesion molecules increased.

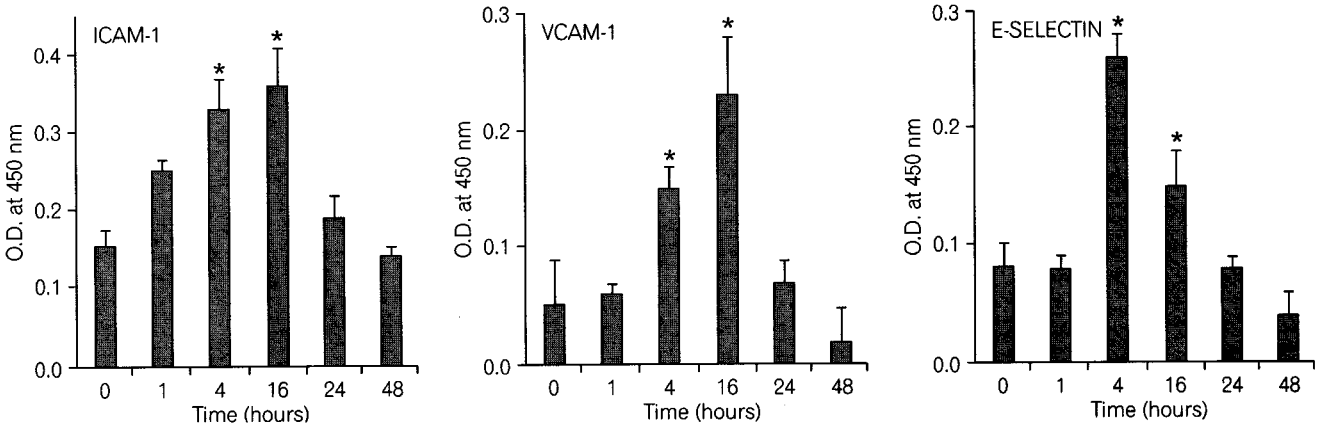

*Fig. 4. Time course of induction of endothelial adhesion molecule expression in response to AECA-positive sera of BD patients. Results show as means ±SD of three observations. *p <0.05 vs. untreated HDMEC*

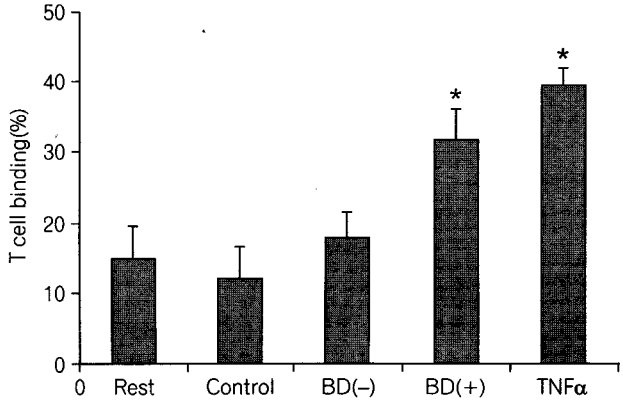

*Fig. 5. The effect of AECA-positive sera of BD patients on T lymphocyte-HDMEC adherence. The percentage of bound T lymphocytes was calculated as: Percent of T lymphocytes binding = (adherence counts-background counts)/counts added per well-background counts) × 100. The results are presented as mean ±SD. *p <0.05 vs. untreated HDMEC.*

selectin was induced at 1 h, peaked at 4 h and had disappeared by 24 h ($p<0.05$) (Fig. 4).

The effect of AECA-positive sera of BD patients on T lymphocyte-HDMEC adherence

In order to determine whether the expression of adhesion molecules on HDMEC correlated functionally with T lymphocyte binding to endothelial cells, we examined the binding of T lymphocytes to monolayers of HDMEC after incubation with AECA-positive sera of BD patients. Binding of T lymphocytes to HDMEC significantly increased from a baseline of 15.0±4.6% to 30.0±4.4% after 16 hours of incubation with AECA-positive sera of BD patients (Fig. 5). AECA-positive sera of BD patients induced time-dependant increases in the binding of T lymphocytes to HDMEC (Fig. 6). The binding of T

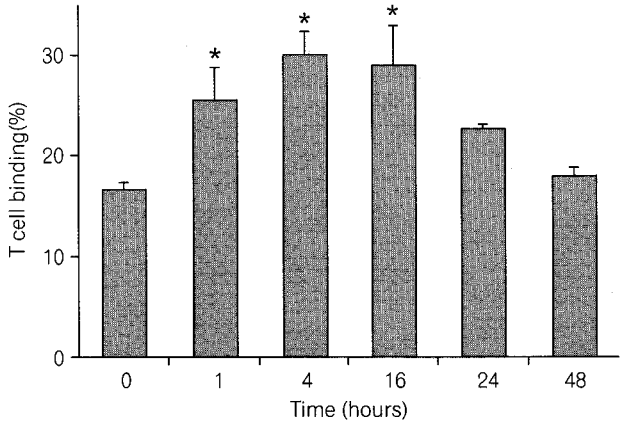

*Fig. 6. Time course of AECA-positive sera of BD patients binding of T lymphocyte to HDMEC monolayers. HDMEC were stimulated with AECA-positive sera of BD patients for 1, 4, 16, 24 and 48 h and then co-incubated with Cr^{51}-labeled T lymphocytes. Data are presented as mean ±SD. *p <0.05 vs. untreated HDMEC.*

lymphocyte to HDMEC significantly increased at 1 h, peaked at 4 h and then slowly decreased to a near basal level at 48 h ($p<0.05$).

DISCUSSION

In this study we have analyzed the effects of AECA-positive sera of BD patients on the capability on stimulating HDMEC to lead to an increase in the expression of EC adhesion molecules on the cell surface and to promote the adherence of T lymphocytes. Pretreatment of human endothelial cells *in vitro* with BD patients' sera containing IgM-AECA led to an increase in the expression of ICAM-1, VCAM-1 and E-selectin molecules, and consequently led to an increase in the adhesion of T lymphocytes. This effect was not found with BD patients' sera that contained no detectable AECA.

Although the pathogenesis of BD still remains obscure, autoantibodies to mucosal cells, the presence of circulating immune complex, changes in the amount of immunoglobulin in serum, and antibody and complement deposition on immunofluorescence all show an autoimmune-mediated pathogenesis of BD.[18-20] Lindquist and Osterland first reported AECA in various inflammatory diseases by indirect immunofluoresence study using a mouse kidney.[21] Lee et al. previously reported 49 (37.4%) out of 131 BD patients had IgM AECA but the antibodies did not increase in normal controls or in other disease control groups.[10] We observed circulating antibodies from sera of BD patients to surface antigens on cultured HDMEC by ELISA. This antibody was not detected in healthy controls.

EC are known targets of BRM. Gamble et al. reported that pretreatment with BRM such as interleukin-1 α (IL-1 α), tumor necrosis factor- α (TNF α), lipopolysaccharide and interferon- γ (IFN- γ) led to an increase or induced the expression of adhesion molecules.[22] Furthermore, activated endothelial cells produced leukocyte chemoattractants and costimulatory signals for lymphocyte activation, and thus initiated or amplified inflammatory injury.[23,24] The expression of ICAM-1 and VCAM-1 on HDMEC can be increased by BRM with the onset of increase at 4 to 6 h and reaching a maximum level at 16 to 24 h.[17] In our study, the expression of ICAM-1 and VCAM-1 started to increase at 1h and reached a peak at 16 h and returned to its base level at 48 h. The expression of E-selectin is rapid with a transient peak at 4 to 8 h after stimulation with BRM and disappears after 24 h.[16] In this study, the induction of E-selectin molecules reached a peak at 4 h and then quickly disappeared. These results showed that stimulation of HDMEC by AECA-positive sera of BD patients caused an increase in the expression of EC adhesion molecules in a similar pattern to stimulation with IL-1 α or TNF α. This interesting effect indicated a direct or indirect action of AECA of BD patients on the regulation of the expression of endothelial cell adhesion molecules.

Bevilacqua et al. reported an increase in T lymphocyte adhesion on HUVEC after stimulation with IL-1 α, TNF α, lipopolysaccharide or IFN- γ.[25] Thornhill et al. reported a similar study on T lymphocyte adhesion and observed an increase in adhesion at 4 h continuing to 72 h after stimulation with BRM.[26] In our study, we observed an increase in the adhesion of T lymphocytes after stimulation of HDMEC with AECA-positive sera of BD patients and found it to be time dependent with an increase in adhesion starting at 1h and continuing until 48 h. We also found that the T lymphocyte adhesion pattern was similar following stimulation with IL-1 α or TNF α.

A recent report by Carvalho et al. showed that an increased adhesion of U937 cells was accompanied by increased expression of endothelial ICAM-1, VCAM-1 and E-selectin molecules.[27] Calvalho et al. suggested that AECA may activate EC by an autocrine or paracrine action of IL-1 α.[27] It can be assumed in our study that AECA acted on HDMEC to induce cytokines such as IL-1 α or TNF α which then activated

EC to the expression of adhesion molecules and thus to induce enhanced T lymphocyte adhesion.

The induction of responses by TNFα suggested the AECA could act on HDMEC to induce the synthesis of a secondary mediator, which then activated the EC. We therefore incubated HDMEC with IgM-depleted sera from AECA-positive sera of BD patients, which failed to show any increase in EC adhesion molecule expression. Although AECA may not directly initiate the inflammatory process, and although the synthesis of a secondary mediator such as IL-1α or TNFα may be involved, this study showed that AECA is needed to actually initiate the disease process.

In summary, the results showed that AECA-positive sera of BD patients are capable of stimulating HDMEC to increase the expression of ICAM-1, VCAM-1 and E-selectin on the cell surface and thereby promote the adherence of T lymphocytes. Although the exact pathogenesis of BD is still unknown, it seems that it involves at least three steps: activation of endothelial cells, adhesion molecule expression, and lymphocyte adhesion. Activation of endothelial cells will facilitate leukocyte traffic and thus initiate an inflammatory injury.

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