Serum levels of IgG antibodies against alpha-enolase are increased in patients with Behçet's disease and are associated with the severity of oral ulcer, erythrocyte sedimentation rates, and C-reactive protein

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ABSTRACT

Objective. *Behçet's disease (BD) is a chronic inflammatory disease of unknown aetiology, characterised by recurrent oral and genital ulcers, skin lesions, uveitis, and arthritis. It is regarded as vasculitis and anti-endothelial cell antibodies (AECA) are found in patients with BD. One of the endothelial cell antibodies was reported to recognise alpha-enolase. This study aimed to investigate expression of alpha-enolase in the surface of peripheral blood cells and serum anti-alpha-enolase antibody (AEA), and their association with clinical manifestations or disease activity of BD.*

Methods. *Cell surface alpha-enolase expression was examined from several cell types of peripheral blood, including lymphocytes, monocytes, and neutrophils using flow cytometry in patients with BD and healthy controls (HCs). IgG AEA levels were measured by enzyme-linked immunosorbent assay (ELISA) in sera from 110 patients with BD, and age/sex matched 110 HCs. Association of alpha-enolase or AEA with clinical manifestation was analysed.*

Results. *The frequency of surface alpha-enolase-expressing cells was increased in BD in lymphocytes and monocytes. Serum AEA levels were increased in BD patients (median [IQR], 0.360 [0.268–0.482], p<0.0001), particularly with mucocutaneous involvement (0.367 [0.273–0.490], p<0.0001) compared to HCs (0.274 [0.231–0.357]). The levels of AEA were correlated with the number of oral ulcer, ESR, and CRP. There was no association between serum levels of AEA and other clinical manifestations.*

Conclusion. *Serum AEA was increased in BD patients and correlated with oral ulcer, ESR and CRP.*

Introduction

Behçet's disease (BD) is a chronic, multisystemic inflammatory disease with clinical manifestations that include recurrent oral and genital ulcers, skin lesions, uveitis, and arthritis (1-3). The aetiology of the disease remains to be elucidated. There are no specific laboratory findings or serological markers of BD, so diagnosis is mainly made based on clinical manifestations (3-4).

Several autoantibodies have been found in patients with BD, including antibodies against endothelial cells, annexin V, heat shock protein (HSP) 70, and C1q (5-6). Among them, anti-endothelial cell antibodies (AECA) are heterogeneous family of antibodies that recognise different proteins on the endothelial cells (7). AECA were examined to determine the characteristics of the autoantigens on the surface of endothelial cells in BD (8). Recently, human alpha-enolase on the surface of endothelial cells has been reported as a target antigen of AECA by a study examining several candidate proteins in BD (8).

Alpha-enolase, a multifunctional protein, is abundant in the cytosol of most cells and is a key glycolytic enzyme. Alpha-enolase is expressed on the surface of many eukaryotic cells after exposure to certain inflammatory stimuli. Human haematopoietic cells, including lymphocytes, monocytes and neutrophils express alpha-enolase on their surfaces in response to exogenous phorbol myristate acetate (PMA) and lipopolysaccharides (LPS) (9). Overexpression of cell-surface alpha-enolase has been reported in infectious diseases, including pneumonia (10), and in several autoimmune diseases, such as rheumatoid arthritis (RA), vasculitis, systemic lu-

pus erythematosus (SLE) and Crohn's disease (9, 11).

The precise role of alpha-enolase in the pathogenesis of BD is still not clear. However, environmental factors, such as bacterial infection (12), hypoxic stress (13), and ultraviolet (UV) light exposure (14), have been shown to induce the expression of alpha-enolase on the cell surface and may lead to formation of autoantibodies that may cause tissue injury (15) in autoimmune disease states (11). There have been several serologic studies of anti-alpha-enolase antibodies (AEA) in several isotypes including IgG or IgM types, in systemic autoimmune and inflammatory diseases (SLE, systemic sclerosis, RA, multiple sclerosis, and BD), and in cancer-associated retinopathy syndrome (16-20).

This study aimed to investigate expression of alpha-enolase in the surface of peripheral blood cells and serum IgG-type anti-alpha-enolase antibody, and their association with clinical manifestations or disease activity of BD.

Materials and methods

Patients and controls

Peripheral blood samples were obtained from patients with 110 BD who fulfilled the classification criteria of the International Study Group for BD (21), and healthy controls (HCs). Clinical information on organ involvement during the 4 weeks prior to blood sampling was prospectively obtained (recurrent oral and genital ulcers, skin lesions such as erythema nodosum and pustule, ocular lesions, and arthralgia). Laboratory measurements including erythrocyte sedimentation rates (ESR) and high sensitivity C-reactive protein (hs-CRP) levels were also examined. Two disease activity questionnaires were administered, the Behçet's Disease Current Activity Form (BDCAF) (22) and the Behçet's Syndrome Activity Score (BSAS). The BDCAF assesses disease activity based on current symptoms and is a composite index of patient and physician assessments of disease scored on a scale of 0 to 12. The BSAS is based solely on patient reports and also assesses current symptoms; it is scored on a scale of 0 to 100. On both questionnaires, higher scores indicate more active disease state. The patient was considered as active disease if BDCAF was ≥2 (23). Analysis of surface alpha-enolase in PBMCs and neutrophils by flow cytometry were conducted at different times in different patients. However there were no significant differences of demographic or clinical manifestations between these patients (data not shown). This study was approved by the Institutional Review Board of Seoul National University Hospital.

Cell isolation

Peripheral blood mononuclear cells (PBMCs) were obtained from anticoagulant-treated blood by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare Bioscience, Uppsala, Sweden). Samples were mixed with an equal volume of phosphate buffered saline (PBS) and then carefully layered on Ficoll. After centrifugation at 890×g for 20 minutes, PBMCs were collected and washed twice with PBS. Neutrophils were taken from the bottom layer following density gradient centrifugation. Red blood cells were lysed by ammonium chloride (StemCell Technologies, Vancouver, Canada) treatment for 10 minutes on ice.

Flow cytometry analysis

Peripheral blood was obtained from 17 patients with BD (48.9±2.8 years) and 12 HCs (31.2±2.3 years) for analysing PBMCs, and 21 BD (47.1±2.2 years) and 10 HCs (43.3±4.0 years) for neutrophils. Isolated cells were resuspended with staining buffer containing 0.5% fetal bovine serum (FBS) (BD Biosciences, San Jose, CA), and non-specific binding was blocked by treatment with human Fc blocking reagent (BD Biosciences) for 10 minutes at room temperature. Then, the samples were stained for 20 minutes on ice in the dark with fluorescent-conjugated antibodies as follows: anti-CD3 peridinin chlorophyll (PerCP) (mouse IgG1,κ, clone SK7), anti-CD11b/Mac-1 phycoerythrin-cyanin7 (PE-Cy7) (mouse IgG1,κ, clone ICRF44), anti-CD14 PE (mouse IgG2b,κ, clone MϕP9), anti-CD16 allophycocyanin (APC) (IgG1,κ, clone B73.1), anti-CD20 PE (mouse IgG1,κ, clone L27) (all from BD Biosciences), and anti-alpha-enolase fluorescein isothiocyanate (FITC) (goat polyclonal) (Santa Cruz Biotechnology, Santa Cruz, CA). Cells were washed twice with staining buffer and analysed using a LSRFortessa instrument and FACSDiva software (all from BD Biosciences). 200,000 events were collected for each analysis. Flow cytometric data were analysed with FlowJo software, v. 10.0.7 (Tree star inc., Ashland, OR). Lymphocytes were gated by size and complexity and monocytes were gated by CD14 positivity from PBMCs. Neutrophils were gated by CD16 and CD11b double-positivity in granulocytes extracted by different gradients from PBMCs.

Western Blot analysis

To identify IgG-type anti-alpha-enolase antibody in the serum, 0.1 or 0.2 μg recombinant human alpha-enolase (Prospec, NessZiona, Israel) was loaded onto a 12% polyacrylamide gel, and electrophoresis was performed. The gel was transferred to a polyvinylidene difluoride membrane. The membranes were blocked overnight at 4ºC with PBS containing 0.05% Tween 20 (PBST) and 3% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO). The sera from BD patients and HCs, diluted 1:100 with PBST containing 1% BSA, were treated as primary antibody and incubated overnight at 4ºC. Rabbit anti-human alpha-enolase antibody (1:1000, Santa Cruz Biotechnology) was used as a positive control. After washing with Tris-buffered saline (TBS) containing 0.1% Tween 20, membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-human IgG antibody (1:5000, Merck-Millipore, Darmstadt, Germany) or HRP-conjugated anti-rabbit antibody (1:5000, Cell Signalling Technology, Inc, Danvers, MA). Immunoreactive bands were visualised with an electrochemiluminescence (Merck-Millipore) detection system (LI-Cor Biosciences, Lincoln, NE).

Enzyme-linked immunosorbent assay (ELISA) of AEA

Sera were obtained from 110 patients with BD (47.5±1.2 years), and 110 age/sex-matched healthy controls (HCs)

Table I. Baseline clinical characteristics of patients with BD and HCs.

	BD patients (n=110)	HCs (n=110)
Age, years (mean ± SEM)	47.5 ± 1.2	47.2 ± 1.6
Sex, female (%)	68 (61.8)	68 (61.8)
Disease duration, years	8.2 ± 0.7	NA
Symptom frequency (%)		
Oral ulcer	87 (79.1)	NA
Genital ulcer	18 (16.4)	NA
Erythema nodosum	44 (40.0)	NA
Pustule	44 (40.0)	NA
Arthralgia	57 (51.8)	NA
Uveitis	8 (7.3)	NA
Enteritis	9 (8.2)	NA
ESR (mm/h)	22.3 ± 1.7	NA
hs-CRP (mg/dL)	0.41 ± 0.07	NA
BDCAF	3.0 ± 0.1	NA
BSAS	25.5 ± 1.4	NA
RF (%)	12 (14.3)	NA
ANA (%)	8 (9.0)	NA
Treatment modality (%)		
Colchicine	77 (70.6)	NA
Steroid	43 (39.4)	NA
Prednisolone equivalent dose (mg/day)	9.5 ± 1.5	NA
Immunosuppressant	44 (40.7)	NA

Immunosuppressants: sulfasalazine, cyclosporine, tacrolimus, azathioprine, or mycophenolate mofetil. NA: not applicable.
BD: Behçet's disease; HC: healthy control; BDCAF: Behçet's Disease Current Activity Form; BSAS: Behçet's Syndrome Activity Score; ESR: erythrocyte sedimentation rate; hs-CPR: high sensitivity C-reactive protein; RF: rheumatoid factor; ANA: anti-nuclear antibody.

(47.2±1.6 years) for measuring IgG or IgM AEA levels by ELISA. Recombinant human alpha-enolase (Prospec) was coated onto microtitre plates (Corning Inc., Corning, NY) with 50 mM sodium carbonate coating buffer overnight at 4ºC. The plates were washed three times with PBST and incubated with PBS containing 1% BSA to block non-specific binding for 1 hour at 37ºC. Then, 100 µl of sera from BD patients and HCs, diluted 1:500 in PBST with 0.1% Triton X-100 (Biosesang, Seongnam, Korea) and 0.5% BSA, were applied to each wells and the plates were incubated for 2 hours at 37ºC. After washing the plates, peroxidase-conjugated goat anti-human IgG antibody (Merck-Millipore) or goat anti-human IgM antibody (Merck-Millipore), diluted 1:5000 in PBST containing 0.5% BSA, was added to each well and the plates were incubated for 1 hour at 37ºC. Antibody binding was detected by the addition of tetramethylbenzidine (TMB) with H_2O_2 (BD Biosciences) as a substrate. The colour reaction was terminated with 2*N* H_2SO_4 (BioLegend, San Diego, CA), and the plates were read spectrophotometrically at a wavelength of 450 nm on an ELISA reader (Bio Tek, Winooski, VT). The levels of AEA were expressed as optical density (OD).

Statistical analysis

Baseline clinical data were presented as mean ± standard error of the mean (SEM). Levels of surface alpha-enolase and serum anti-alpha-enolase antibody were presented as median (interquartile range [IQR]: 25th percentile to 75th percentile). Nonparametric Mann-Whitney U-tests and Kruskal-Wallis tests were performed to determine significant differences between groups. A *p*-value less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS, v. 21 (SPSS Inc., Chicago, IL). Graphs were created using Origin software, v. 9.1 (OriginLab Corp., Northampton, MA).

Results

Baseline clinical characteristics

The clinical and laboratory findings in 110 patients with BD are shown in Table I. The mean age of patients was 47.5 years old, 61.8% were females. Disease duration after BD diagnosis was 8.2 years. The mean levels of ESR and CRP were 22.3 mm/h and 0.4 mg/dL. The mean patient index score of BDCAF and BSAS were 3.0 and 25.5, respectively. Seventy-seven (70.6%) patients were on colchicine, forty-four (40.7%) patients were on at least one immunosuppressant medication (sulfasalazine, cyclosporine, tacrolimus, azathioprine, or mycophenolate mofetil), and forty-three (39.4%) were on steroids.

Increased expression of cell surface alpha-enolase in PBMCs from patients with BD

We examined alpha-enolase expression in the membrane of peripheral blood cells from 17 BD patients and 12 HCs using flow cytometry (Fig. 1). PBMCs from BD patients had higher expression of alpha-enolase on the cell surface than those from HCs (Fig. 1A). Then we examined surface alpha-enolase levels in lymphocytes, monocytes (BD, n=17; HC, n=12) and neutrophils (BD, n=21; HC, n=10) from the peripheral blood of BD patients and HCs (Fig. 1B). On the surface of lymphocytes and monocytes, the expression levels of alpha-enolase were significantly elevated in patients with BD compared to HCs (median [IQR], 1.84 [1.47–2.33]% *vs*. 1.15 [0.89–1.33]%, p=0.003 and 6.75 [5.65–9.52]% *vs*. 3.07 [2.42–3.80]%, p=0.008, respectively). When lymphocytes were subdivided into T cell ($CD3^+$) and B cell ($CD20^+$), there was significant increase in the expression of alpha-enolase in each cell subsets in BD compared to HCs (1.69 [1.20–2.0]% *vs*. 0.95 [0.80–1.09]%, p<0.0001 and 4.52 [2.84–6.05]% *vs*. 2.55 [1.90–2.87]%, p=0.009, respectively) (Fig. 1C).

Correlation between surface expression of alpha-enolase on PBMCs and clinical or laboratory parameters in patients with BD

The expression of alpha-enolase in lymphocytes, monocytes or neutrophils was analysed to determine the association with the clinical manifestations or disease activity in BD patients (Table II). Expression of alpha-enolase in lymphocytes was correlated with ESR.

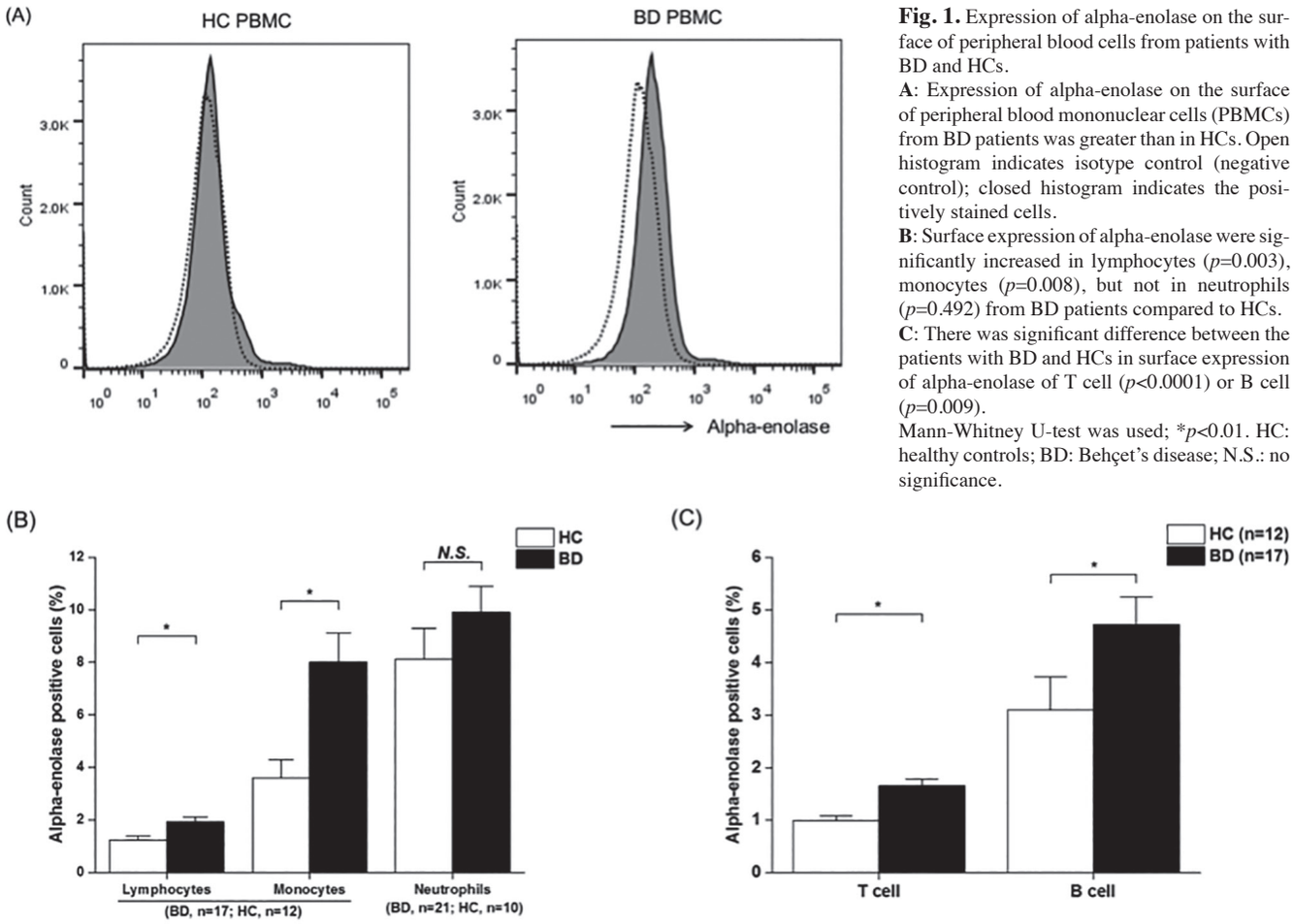

Fig. 1. Expression of alpha-enolase on the surface of peripheral blood cells from patients with BD and HCs.
A: Expression of alpha-enolase on the surface of peripheral blood mononuclear cells (PBMCs) from BD patients was greater than in HCs. Open histogram indicates isotype control (negative control); closed histogram indicates the positively stained cells.
B: Surface expression of alpha-enolase were significantly increased in lymphocytes (p=0.003), monocytes (p=0.008), but not in neutrophils (p=0.492) from BD patients compared to HCs.
C: There was significant difference between the patients with BD and HCs in surface expression of alpha-enolase of T cell (p<0.0001) or B cell (p=0.009).
Mann-Whitney U-test was used; *p<0.01. HC: healthy controls; BD: Behçet's disease; N.S.: no significance.

When lymphocytes were divided into T cells and B cells (Fig. 2), alpha-enolase expression in T cells had correlation with the ESR (p=0.006, Fig. 2A) and tended to be correlated with CRP (p=0.051, Fig. 2B). However, alpha-enolase expressed monocytes or neutrophils had no correlation with clinical manifestations or disease activity.

The levels of AEA in sera from patients with BD versus HCs

To identify IgG-type AEA from serum, western blotting was performed. Recombinant human alpha-enolase was applied to the membrane, which was then incubated with sera from BD patients or HCs and treated with HRP-conjugated anti-human IgG antibody. In contrast to the HC sera, a specific band at 48 KDa corresponding to the human alpha-enolase protein was detected in sera from BD patients (Fig. 3A). Quantitative analysis of serum AEA was conducted by ELISA. BD patients showed increased levels of IgG AEA compared to HCs (0.360 [0.268–0.482] *vs*. 0.274 [0.231–0.357], p<0.0001) (Fig. 3B). Then we divided the BD patients to active and inactive disease according to the score of BDCAF, active patients showed higher levels of AEA than did inactive (0.375 [0.274–0.537] *vs*. 0.284 [0.209–0.371], p<0.032) (Fig. 3C). We also measured the IgM AEA in identical samples to compare with the IgG AEA, but there was no difference of IgM AEA in the sera from BD compared to HCs (0.538 [0.351–0.763] *vs*. 0.504 [0.344–0.823], p=0.764) and no association of AEA levels and clinical manifestations in patients with BD (data not shown).

Table II. Correlation between surface expression of alpha-enolase in lymphocytes, monocytes, and neutrophils and clinical manifestations.

Parameter	Lymphocytes (n=17)		Monocytes (n=17)		Neutrophils (n=21)	
	correlation coefficient	p-value	correlation coefficient	p-value	correlation coefficient	p-value
Number of OU	0.133	0.623	0.008	0.978	-0.227	0.323
Number of GU	-0.464	0.070	-0.474	0.064	-0.268	0.240
Number of EN	0.395	0.130	0.207	0.443	0.148	0.523
Number of PU	0.120	0.658	-0.369	0.160	0.185	0.423
ESR	0.585	0.017*	0.235	0.380	0.173	0.492
hs-CRP	0.456	0.088	0.463	0.082	0.147	0.588
BDCAF	0.251	0.348	-0.124	0.648	-0.060	0.809
BSAS	0.171	0.527	-0.105	0.700	-0.023	0.925

Spearman's rho was used; *p<0.05. OU: oral ulcer; GU: genital ulcer; EN: erythema nodosum; PU: pustule; BDCAF: Behçet's Disease Current Activity Form; BSAS: Behçet's Syndrome Activity Score; ESR: erythrocyte sedimentation rate; hs-CPR: high sensitivity C-reactive protein.

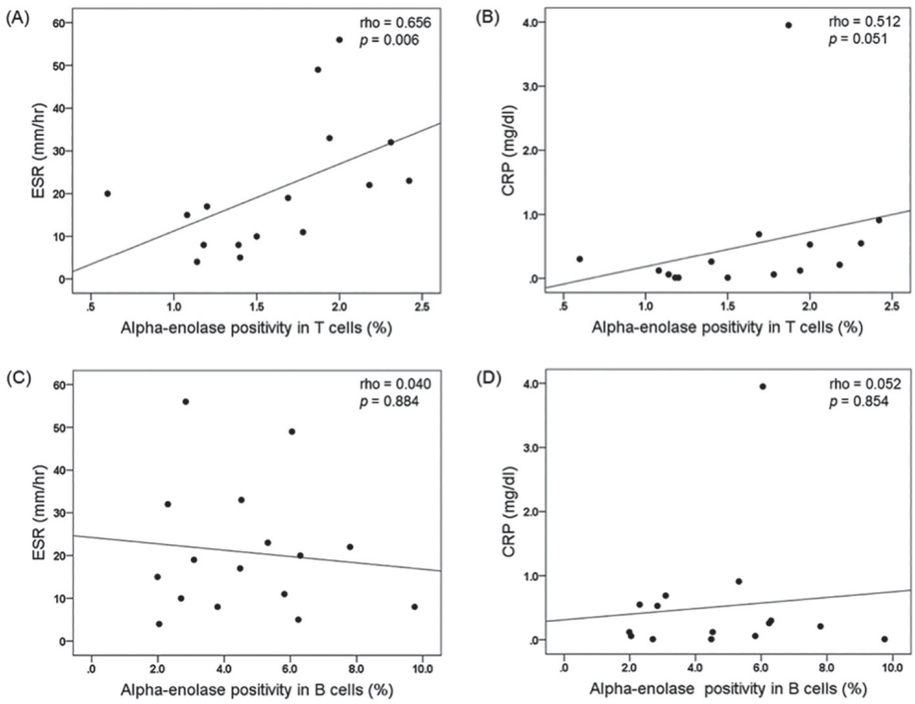

Fig. 2. Correlation between expression of alpha-enolase on T cells or B cells and ESR or CRP.
A: Expression of alpha-enolase in T cells was correlated with the levels of ESR (**A**) and **B**: tended to be correlated with CRP.
C-D: Levels of ESR or CRP were not correlated with expression of alpha-enolase in B cells.

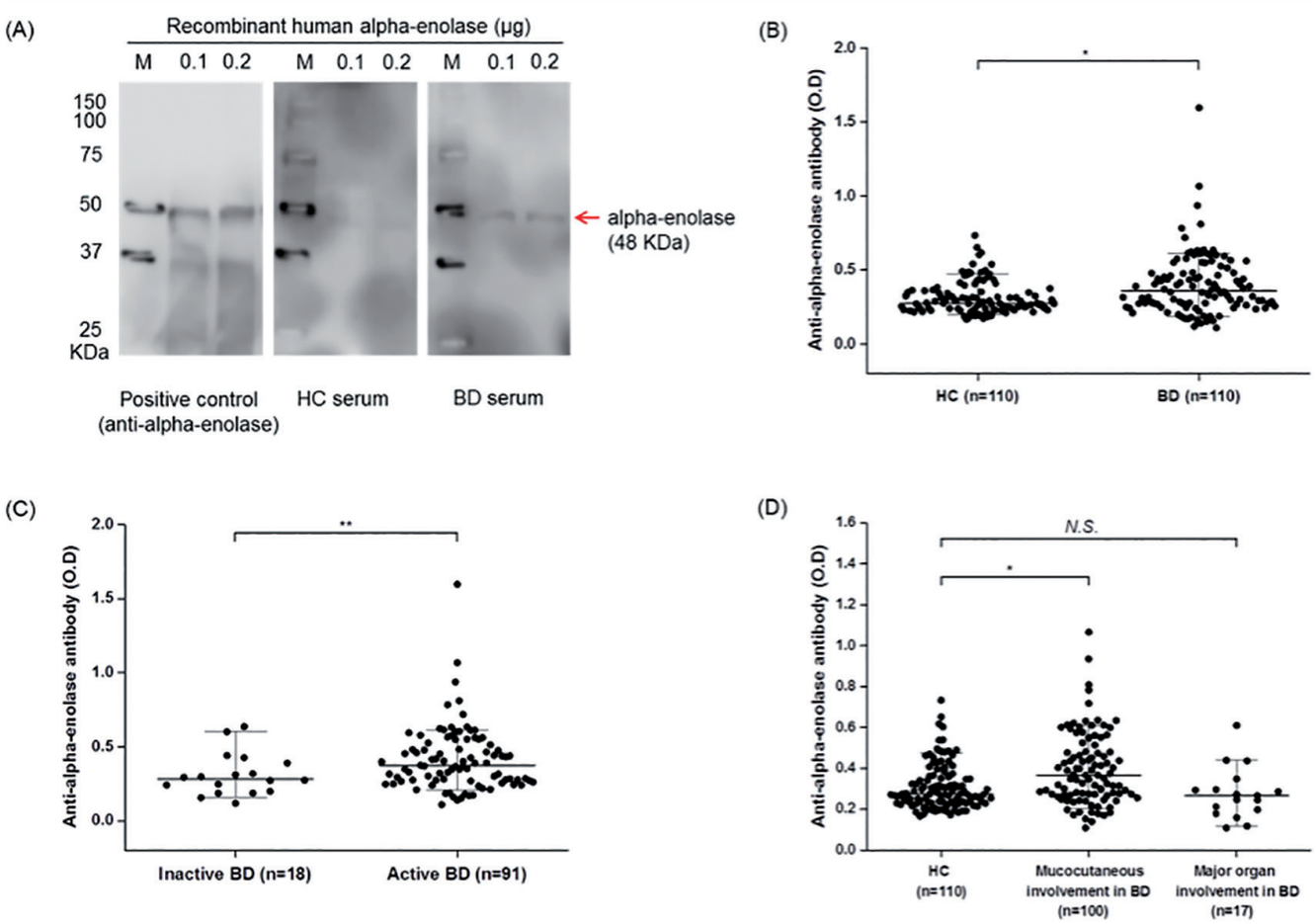

Fig. 3. **A**: Serum levels of anti-alpha-enolase antibody in BD patients and HCs. **B**: Representative immunoblot of serum AEA was detected in the positive control and BD patient. **C**: Quantitative analysis of serum AEA levels were measured by ELISA assay in HC and BD. Patients with BD were divided according to the BDCAF. **D**: or mucocutaneous involvement and major organ involvement.
The lines in the middle indicate the median; the bars indicate the 10th and 90th percentiles. Mann-Whitney U-test was used; *p<0.01, **p<0.05. M: marker; HC: healthy controls; BD: Behçet's disease.

Table III. Comparison of the mean levels of AEA in BD patients with the presence or absence of clinical manifestations.

Parameter	Number of patients	AEA [median (IQR)]	*p*-value
Healthy controls		0.274 (0.231-0.357)	
Oral ulcer	No (n=23)	0.247 (0.172-0.339)	0.001*
	Yes (n=87)	0.398 (0.278-0.537)	
Genital ulcer	No (n=92)	0.351 (0.264-0.516)	0.840
	Yes (n=18)	0.406 (0.274-0.457)	
Erythema nodosum	No (n=66)	0.319 (0.251-0.462)	0.101
	Yes (n=44)	0.402 (0.277-0.531)	
Pustule	No (n=66)	0.389 (0.275-0.550)	0.188
	Yes (n=44)	0.301 (0.254-0.455)	
Uveitis	No (n=102)	0.368 (0.270-0.522)	0.107
	Yes (n=8)	0.285 (0.204-0.371)	
Arthralgia	No (n=53)	0.320 (0.272-0.426)	0.157
	Yes (n=57)	0.390 (0.261-0.564)	
Enteritis	No (n=96)	0.372 (0.274-0.516)	0.013**
	Yes (n=9)	0.247 (0.198-0.286)	
Rheumatoid factor	No (n=71)	0.372 (0.254-0.481)	0.692
	Yes (n=12)	0.341 (0.268-0.458)	
Anti-nuclear antibody	No (n=81)	0.389 (0.274-0.550)	0.636
	Yes (n=7)	0.326 (0.245-0.428)	

Mann-Whitney U-test was used; *p<0.01, **p<0.05.
BD: Behçet's disease; AEA: anti-alpha-enolase antibody; IQR: interquartile range.

Table IV. Correlation between serum anti-alpha-enolase antibody and clinical manifestations or disease activity.

Parameter	Correlation coefficient	*p*-value
Number of oral ulcers	0.260	0.006*
Number of genital ulcers	0.010	0.917
Number of erythema nodosums	0.162	0.092
Number of pustules	-0.130	0.176
ESR	0.315	0.001*
hs-CRP	0.246	0.013**
BDCAF	0.186	0.053
BSAS	0.116	0.234

Spearman's rho was used; *p<0.01, **p<0.05. BDCAF: Behçet's Disease Current Activity Form; BSAS: Behçet's Syndrome Activity Score; ESR: erythrocyte sedimentation rate; hs-CPR: high sensitivity C-reactive protein.

Correlation of serum AEA with clinical manifestations in patients with BD

We divided the patients with BD according to the presence or absence of clinical manifestations (Table III). BD patients with oral ulcer had higher levels of AEA than the BD patients without oral ulcer and the HCs (Kruskal-Wallis test p<0.0001). In the patients groups with enteritis, the AEA levels were low, but there was no difference between the group of patients and HCs (Mann-Whitney U-test p=0.191). Other clinical manifestations including genital ulcer, erythema nodosum, and arthralgia tended to show increased levels of AEA in BD with manifestations, but the differences were not statistically significant. We also organised the group as the mucocutaneous involvement or major organ involvement (Fig. 3D). The group of mucocutaneous involvement consisted of patients with symptoms including oral ulcer, genital ulcer, erythema nodosum or pustules. The group of major organ involvement consisted of patients with symptoms including uveitis or enteritis. In the group of BD with mucocutaneous involvement, the levels of AEA were higher than HCs (0.367 [0.273–0.490] *vs.* 0.274 [0.231–0.357], p<0.0001). When we compared the group of patients with major organ involvement with HCs, there was no difference between two groups (0.267 [0.198–0.297] *vs.* 0.274 [0.231–0.357], p=0.248). There was no difference in serum AEA levels according to medication (colchicine, steroid, or immunosuppressant) (data not shown). We analysed the correlation between the serum AEA levels and severity of clinical manifestations, laboratory parameters, or disease activity in BD patients (Table IV). Serum level of AEA was positively correlated with the number of oral ulcer (rho=0.260, p=0.006), ESR (rho=0.315, p=0.001), and CRP (rho=0.246, p=0.013) and it tended to be correlated with the score of BDCAF (rho=0.186, p=0.053).

Discussion

AECA have been detected in diseases associated with vascular injury, such as systemic vasculitis, RA, and BD (5, 24). The characteristics of the antigens that bind AECA are not well understood because AECA targets heterogeneous proteins on the surface of endothelial cells (7). Recently, alpha-enolase was reported to be one of the target antigens of AECA in BD (8). They investigated the characteristics of alpha-enolase on the surface of endothelial cells and its serum antibody levels. The levels of serum IgM-type AEA were increased in BD patients with ocular lesions or thrombosis. In intestinal BD, a type of inflammatory bowel disease (IBD), levels of IgM AEA were increased and were correlated with the scores of the Harvey-Bradshaw index (HBI), the disease severity index used in assessing Crohn's disease (25).

Enolase, a multifunctional protein, is abundantly found in the cytosol of most cells. In mammals, the enzyme has 3 subunits, α, β and γ, each encoded by a separate gene, which can combine to form homodimers or heterodimers, thereby creating different isoenzymes

(11). In human cells, the three homodimeric forms are common (αα, or enolase-1, ββ, or enolase-3, and γγ, or enolase-2). The isoenzymes are found in different locations and have different functions. Alpha-enolase exists ubiquitously throughout the various cells (11), beta-enolase is mainly found in muscle as a muscle specific enolase (26), and gamma-enolase is neuron-specific (27). Alpha-enolase, which is expressed in most tissues, catalyses the dehydration of 2-phospho-D-glycerate to phosphoenolpyruvate during the ninth step of glycolysis. Besides its well-known glycolytic role, alpha-enolase has diverse functions and is located in many different sites, so it may affects various biological and pathophysiological processes (11). Tau-crystallin, a major lens protein in vertebrates, shows a similarity with the sequence of human alpha-enolase (28). The alpha-enolase gene can be alternatively spliced into a nuclear form, Myc-binding protein-1, which inhibits the c-myc protooncogene (29). In addition, alpha-enolase was identified as a heat shock protein in Saccharomyces cervesiae (30).

Alpha-enolase can exist in the surface of a variety of cells as a plasminogen-binding receptor (31). Some activated immune cells, stimulated by PMA or LPS, and some tumur cells express alpha-enolase on the cell membrane (9, 32). Plasminogen binds to alpha-enolase and then is converted to plasmin, the activated form of plasminogen, which enhances fibrinolysis and contributes to tissue invasion, metastasis, and so on (32). Some pathogens, such as *Streptococcus pneumoniae*, apply that system to invade host cells (33), so patients with infectious diseases, such as pneumonia have increased alpha-enolase on their cell surface of blood monocytes (10). In addition, increased alpha-enolase on the cell surface has been reported in several autoimmune diseases, including RA, SLE, vasculitis, and Crohn's disease (9, 11). Surface expressed alpha-enolase on the peripheral immune cells can induce autoantibody production. We analysed several types of immune cells to investigate the expression of surface alpha-enolase for the following reasons: lymphocytes are activated in patients of BD (34); various proinflammatory cytokines are produced by monocytes such as interleukin-1 (IL-1), IL-6, and tumour necrosis factor-α (TNF-α) (35); and neutrophils are hyperactive in BD (36).

We found significantly increased levels of alpha-enolase on the surface of lymphocytes and monocytes but not in neutrophils in patients with BD compared to HCs. Moreover the levels of alpha-enolase in lymphocytes, especially in T cells, were correlated with the ESR levels of patients with BD.

Then we investigated serum IgG-type AEA. Unlike IgM that engage in first-line of defense against infection, IgG is the main type of antibody in adaptive immune response and occupy about 75% of serum antibodies. Previous report indicated that IgG AEA were detected more frequently in patients with various systemic autoimmune disorders, such as SLE, and systemic sclerosis than in HCs (15), but its levels had not yet been reported in patients with BD.

In the present study, the levels of IgG-type AEA were increased in patients with BD. We also found a correlation between serum levels of antibody and the number of oral ulcer, the level of ESR or CRP. In previous studies, IgM AEA was associated with the involvement of the vascular system in BD (8, 25). However, our data showed that the levels of IgM AEA did not differ between sera from BD and HCs. Actually there was no BD patient with vascular involvement such as thrombophlebitis, deep vein thrombosis, or cardiovascular symptoms in our study. This may imply that different isotypes of AEA may have different functions depending on inflammation site.

The precise role of alpha-enolase in the pathogenesis of BD is still not clear. However, environmental factors, such as bacterial infection (12), hypoxic stress (13), and UV exposure (14), have been shown to induce the expression of alpha-enolase on the cell surface and may lead to formation of autoantibodies that can cause endothelial injury (15) in several disease states (11).

In previous study, serum alpha-enolase levels were increased in patients with BD and recurrent aphthous stomatitis (RAS) compared to HCs (37). Our study showed that surface expression of alpha-enolase in T cells was increased in BD and correlated with the levels of ESR. Moreover serum AEA levels were increased in patients with BD and correlated with oral ulcer, the levels of ESR and CRP. These results suggest that surface expressed alpha-enolase may be increased in the inflammatory condition and contribute to the formation of its autoantibody. It remains to be elucidated what kind of stimulus upregulate the alpha-enolase in the surface of immune cells and what is the role of AEA in pathogenesis of BD. We did not have patients with vascular or neurologic involvement. Further study may be needed in a large scale to confirm the correlation of AEA with various organ involvement.

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