
Human anti- α -enolase antibody in sera from patients with Behçet's disease and rheumatologic disorders

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ABSTRACT

Objective. α -Enolase is a target antigen of IgM-type anti-endothelial cell antibody in patients with Behçet's disease (BD). The objective of this study was to assess the reactivity of serum anti- α -enolase antibodies in BD and in other rheumatologic diseases, and to evaluate the clinical significance of serum anti- α -enolase antibodies in BD.

Methods. Enzyme-linked immunosorbent assay (ELISA) and immunoblotting were used to examine serum samples from patients with BD (n=100), systemic lupus erythematosus (SLE) (n=50), systemic sclerosis (n=21), rheumatoid arthritis (RA) (n=20), Takayasu's arteritis (n=20), dermatomyositis (n=17), mixed connective tissue disease (MCTD) (n=11), and samples from healthy volunteer donors (n=23). The medical records of patients with BD were reviewed to investigate their clinical characteristics.

Results. Specific positive signals against recombinant human α -enolase were detected by IgM ELISA of serum samples from 56 of the 100 BD patients (56.0%), 24 of the 50 SLE patients (48.0%), 15 of the 21 systemic sclerosis patients (71.4%), 13 of the 20 RA patients (65.0%), 10 of the 20 Takayasu's arteritis patients (50.0%), 9 of the 17 dermatomyositis patients (52.9%), and 5 of the 11 MCTD patients (45.5%). The number of BD patients with vascular lesions was significantly higher in the anti- α -enolase antibody positive group than in the negative group (p=0.027).

Conclusions. We demonstrated the reactivities of serum anti- α -enolase antibodies in BD and other rheumatologic diseases with moderate specificity and also found that serum anti- α -enolase antibodies in BD can be associated with vascular system involvement.

Introduction

Behçet's disease (BD) is a chronic multisystemic vasculitis that mainly affects small blood vessels. The pathogenesis of BD remains unclear (1), however, several autoantibodies have been identified, including anti-kinectin antibodies, anti- α -tropomyosin antibodies, and anti- α -enolase antibodies (2, 3). Lee *et al.* identified α -enolase as a target antigen of IgM-type anti-endothelial cell antibody (AECA) in patients with BD (3). However, the pathogenetic role of anti- α -enolase antibody in BD has not been fully elucidated. Moreover, several studies show association between antibodies against α -enolase and various inflammatory and immune disorders including anti-neutrophil cytoplasmic antibody-positive vasculitis, inflammatory bowel disease, systemic lupus erythematosus (SLE), systemic sclerosis, primary membranous nephropathy, mixed connective tissue disease (MCTD), cancer-associated retinopathy, autoimmune liver diseases, Kawasaki disease, and severe asthma (4-8). In this study, we compared the positive reactivity of serum anti- α -enolase antibodies in BD with that in other rheumatologic diseases, including SLE, systemic sclerosis, rheumatoid arthritis (RA), Takayasu's arteritis, dermatomyositis, and MCTD. We also evaluated the clinical significance of serum anti- α -enolase antibodies in BD by reviewing medical records and the results of laboratory tests.

Materials and methods

Patients

This study was performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki with approval from the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine, Seoul, Korea. After obtaining informed consent, we col-

lected serum samples from 100 patients with BD, who were diagnosed based on criteria outlined by the International Study Group for BD and the revised criteria of the BD Research Committee of Japan (9, 10). We reviewed the medical records of patients with BD to investigate their personal and clinical characteristics and laboratory test results, including full blood count, blood glucose, renal and liver function tests, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), anti-streptolysin O (ASO), rheumatoid factor (RF), antinuclear antibodies (ANA), and venereal disease research laboratory (VDRL) tests. Vascular system involvement of BD was determined based on the clinical manifestations, serologic tests, and radiologic tests. Imaging test included angiogram, computed tomography, and Doppler ultrasonography. Serum samples from patients with SLE (n=50), systemic sclerosis (n=21), RA (n=20), Takayasu's arteritis (n=20), dermatomyositis (n=17), and MCTD (n=11) were collected and diagnoses of the rheumatologic disorders were made by two rheumatologists according to clinical findings and results of serologic tests. Serum samples were also obtained from 23 healthy volunteer donors. The serum samples were stored at -70°C prior to testing for anti- α -enolase antibodies.

Separation and purification of human α -enolase antigen

Briefly, cDNA encoding human α -enolase was prepared as described previously (3), and recombinant plasmid DNA carrying an α -enolase-Glutathione-S-transferase fusion construct was introduced into *Escherichia (E.) coli* DH5 α cells. Transformed *E. coli* DH5 α cells were cultured on Luria-Bertani (LB) agar supplemented with ampicillin for 16 hours and a single colony was cultured in LB broth until the absorption density at 600 nm reached 0.45-0.55 as measured by colorimetry. To induce expression of recombinant protein, 1 mM isopropyl- β -D-thiogalactopyranoside was added followed by incubation for 16 hours at 25°C. Glutathione S-transferase fusion protein in the supernatants was adsorbed with glutathione-Sepharose 4B beads and human α -enolase was purified by treatment with thrombin protease.

Enzyme-linked immunosorbent assay (ELISA)

IgM ELISA was performed using recombinant human α -enolase antigen. A 96-well microtiter plate (Immuno2, HB, Thermo Scientific, Waltham, MA) was coated overnight with 250 ng recombinant human α -enolase antigen. The plate was washed three times with 0.05% phosphate-buffered saline-Tween 20 (PBST) to block non-specific binding. Then, 100 μ L of serum from BD patients, patients with rheumatologic disorders, and normal controls, diluted 1:20 in PBST containing 1% bovine serum albumin (Sigma, St Louis, MO), was added to each well, and the plate was incubated for 1 hour at 37°C.

After washing the plate three times with PBST, quantification of antibody binding was performed colorimetrically by addition of substrate (tetramethylbenzidine, Sigma) to each well. Optical density (OD) of the plates was read spectrophotometrically at 450 nm on an ELISA reader (Dynatech, Alexandria, VA) and positivity was defined as an OD value greater than three standard deviations (SD) above the mean value of normal controls.

Immunoblot analysis

After mixing purified recombinant human α -enolase (3 μ g) with the same volume of sample buffer, the samples were loaded onto a 10% polyacrylamide gel and subjected to electrophoresis at 100V. Protein was transferred to a nitrocellulose membrane at 150 mA 10-15V, and the membrane was washed with PBST and incubated for 1-3 hours at room temperature in blocking buffer (5% non-fat dry milk, 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20). The membrane was then incubated with gentle agitation for 1 hour at room temperature with serum samples from normal controls or patients with BD or rheumatologic disorders, diluted 1:20 with a primary antibody dilution buffer. Goat anti-human α -enolase antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a positive control at a dilution of 1:100. The membrane was washed three times with PBST and incubated at room temperature for 2 hours with peroxidase-conjugated goat anti-human IgM antibody. After washing three times with PBST,

3,3-diaminobenzidine tetrahydrochloride (Sigma) containing 30% H₂O₂ was added and the membrane was incubated at 37°C for 10 minutes.

Statistical analysis

Chi-square test, Fisher's exact test, and multiple logistic regression were applied to assess differences in the clinical features of BD patients with positive and negative reactivity for recombinant human α -enolase. All analyses were performed using Statistical Package for the Social Sciences version 11.0 (SPSS Inc., Chicago, IL). Differences were considered statistically significant when the *p*-value was less than 0.05.

Results

Reactivity of anti- α -enolase antibody

In ELISA assays for anti- α -enolase antibody, the mean OD (\pm 1 SD) for serum samples from the 23 healthy controls was 0.139 \pm 0.0682. An OD exceeding this value by \geq 3 SD was defined as positive reactivity. The results of ELISA for detection of anti- α -enolase antibody in serum samples from patients with BD, SLE, systemic sclerosis, RA, Takayasu's vasculitis, dermatomyositis, and MCTD are summarized in Figure 1.

Specific positive signals against recombinant human α -enolase were detected in serum samples from 56 of the 100 BD patients (56%). In samples from patients with other systemic rheumatic diseases, anti- α -enolase antibodies were detected in 24 of the 50 SLE patients (48%), 15 of the 21 systemic sclerosis patients (71.4%), 13 of the 20 RA patients (65%), 10 of the 20 Takayasu's arteritis patients (50%), nine of the 17 dermatomyositis patients (52.9%), and five of the 11 MCTD (45.5%) patients (Table I). Serum samples from all of the patients that exhibited positive reactivity on ELISA were also shown to have specific bands positive for recombinant human α -enolase in immunoblot analysis, whereas no reactivity was observed in samples from patients with negative reactivity and normal controls.

Clinical significance of anti- α -enolase antibodies in patients with BD

Among the 100 BD patients, 56 patients (21 males and 35 females, mean age 34.9 \pm 10.2) showed positive reactivity with recombinant human α -enolase and

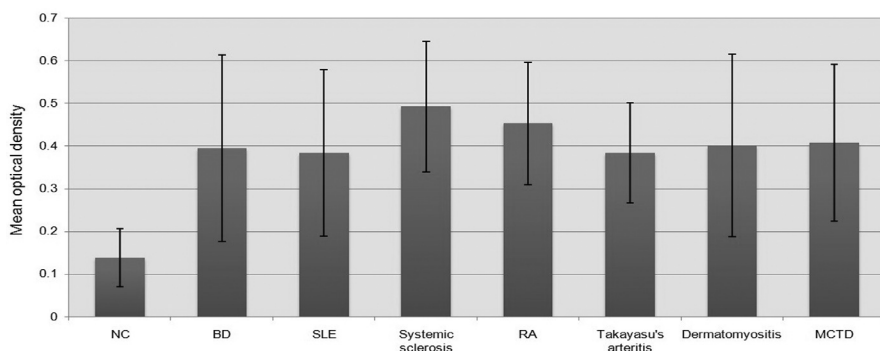


Fig. 1. Mean optical density of ELISA for detection of anti- α -enolase antibody in serum samples from normal controls (NL) and patients with Behçet's disease (BD), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), dermatomyositis, systemic sclerosis, mixed connective tissue disease (MCTD), and Takayasu's arteritis. Vertical error bars show standard deviation.

Table I. Reactivity of anti- α -enolase antibody obtained from normal control subjects, patients with Behçet's disease, and patients with other rheumatologic diseases.

Group	No. of subjects	No. (%) of subjects positive for anti- α -enolase antibody
Normal control subjects	23	0 (0.0)
Behçet's disease patients	100	56 (56.0)
Systemic lupus erythematosus patients	50	24 (48.0)
Systemic sclerosis patients	21	15 (71.4)
Rheumatoid arthritis patients	20	13 (65.0)
Takayasu's arteritis patients	20	10 (50.0)
Dermatomyositis patients	17	9 (52.9)
Mixed connective tissue disease	11	5 (45.5)

44 patients (16 males and 28 females, mean age 34.6±9.5) showed negative reactivity.

In BD patients with positive reactivity for recombinant human α -enolase, the following symptoms were observed in descending order of frequency: recurrent oral ulcers in 56 patients (100%), skin lesions in 51 (91.1%), genital ulcers in 49 (87.5%), articular involvement in 25 (44.6%), ocular involvement in 23 (41.1%), vascular involvement in 12 (21.4%), and gastrointestinal lesions in four (4.1%). None of the patients showed central nervous system involvement.

In BD patients with negative reactivity for recombinant human α -enolase, BD-related symptoms were observed as follows: recurrent oral ulcers and skin lesions in all 44 patients (100%), genital ulcers in 40 (86.4%), articular involvement in 25 (56.8%), and ocular involvement in 20 (45.5%). Vascular involvement, gastrointestinal lesions, and central nervous system involvement were each observed in two patients (4.5%).

The number of BD patients diagnosed with BD-related vascular lesions was significantly higher in the anti- α -enolase

antibody positive group than in the negative group (21.4% vs. 4.5%, $p=0.027$, OR=5.92, 95% confidence interval 1.23-28.49). However, there were no statistically significant differences in any other clinical features of BD and clinical types between the anti- α -enolase antibody positive and negative groups. Furthermore, the results of laboratory tests, including full blood count, blood glucose, renal and liver function tests, ESR, CRP, ASO, RF, ANA, and VDRL, were all similar between the anti- α -enolase antibody positive and negative groups.

Discussion

The glycolytic enzyme α -enolase is usually located in the cytoplasm but can be expressed in the cell membrane of eukaryotic cells, including monocytes, T cells, B cells, neuronal cells, and endothelial cells, following inflammatory stimulus through unknown mechanisms (3, 4, 11). Testing for antibodies to α -enolase has been proposed as a diagnostic tool or a biological marker for various conditions, including BD, Kawasaki's disease, RA, and severe asthma (3, 7, 8, 12). However, the precise role of α -enolase in the

pathogenesis of BD is not clear and the sensitivity and specificity of antibodies against α -enolase in various diseases remain to be elucidated. Identification of autoantibodies to α -enolase can be interpreted as an epiphenomenon reflecting epithelial or endothelial cell damage secondary to chronic inflammation, or an autoimmune response to α -enolase as a pathogenic factor inducing inflammation (8).

Inflammatory diseases of blood vessel wall, including BD, Kawasaki's disease, Takayasu's arteritis, and giant cell arteritis, characteristically demonstrate endothelial proliferation, fibrosis and thrombus formation, which eventually result in tissue ischemia (13). Enolase functions as the plasminogen receptor on the surface of various cells and plays a crucial role in fibrinolysis by binding of plasminogen to α -enolase. It has been suggested that α -enolase, especially expressed on the cell surface of endothelial cells and reacting with plasminogen, can have an important role in the initiation of the disease process by modulating the pericellular and intravascular fibrinolytic system (3, 4).

In this study, reactivity against recombinant human α -enolase in the sera of BD, SLE, systemic sclerosis, RA, Takayasu's arteritis, dermatomyositis, and MCTD patients was 56%, 48%, 71.4%, 65%, 50%, 52.9% and 45.5%, respectively. Patients with BD, SLE, and RA enrolled in our study exhibited higher reactivity than in previous reports (3, 12, 14). According to the study by Lee *et al.*, reactivity against recombinant human α -enolase in IgM ELISA of serum samples from 40 BD, 6 RA, 5 SLE, and 2 Wegener's granulomatosis patients was 37.5%, 16.7%, 0%, and 100%, respectively (3). Mosca *et al.* reported reactivity against recombinant human α -enolase by IgM ELISA in serum samples from 14 of 68 (21%) randomly selected 68 SLE patients (14). In patients with RA, antibodies against citrullinated α -enolase were detected in 24 of 52 RA patients (46%) and α -enolase was also detected in the joints of RA patients (12).

Although the clinical significance of anti- α -enolase antibodies in patients with BD is not clear, several reports have described the clinical characteristics of BD patients positive for AECA

(15, 16). One study demonstrated that 13 of 72 Turkish BD patients (18.1%) were positive for AECA, and that AECA-positive BD patients exhibited a higher frequency of vascular lesions (15). Also, Cervera *et al.* found that 15 of 30 BD patients (50%) were positive for AECA and that positivity for AECA was associated with active ocular lesions (16). In RA, AECA titers are related to the severity of vasculitis (17). Moreover, SLE patients with an active lesion of the kidney and vasculitis presented high titers of AECA, and it has been suggested that the AECA titer could be used as a measure of renal involvement (18). Several mechanisms have been proposed by which AECAs may play a role in the pathophysiology of inflammatory diseases (19-21). Binding of AECA to endothelial cell may result in activation of endothelial cell, which may be associated with increased secretion of chemoattractants and/or cytokines as well as secretion or inhibition of prostacyclin (19, 20). AECAs could also trigger the inflammatory processes by complement dependent cytotoxicity and/or antibody dependent cellular toxicity (21). In this study, we observed that the number of BD patients with vascular lesions was significantly higher in the anti- α -enolase antibody positive group than in the negative group. However, the other clinical features of BD, including ocular lesions, were not significantly associated with positivity for anti- α -enolase.

Although the precise pathogenesis of BD remains obscure, several reports suggest involvement of *streptococci* in BD (22-24). Antibody against human α -enolase from patients with BD was shown to have cross-reactivity with an antigen of *Streptococcus sanguis* that was subsequently identified as streptococcal α -enolase (23). Streptococcal antibody tests, such as ASO and anti-DNase B, are generally used for the diagnosis of streptococcal infections. BD patients with persistently high ASO titers are known to have a more frequent history of tonsillitis and erythema nodosum-like lesions than BD patients with normal ASO titer (24). In our study, we did not observe a significant difference

in ASO titer levels between the anti- α -enolase antibody positive and negative groups. However, because elevated ASO titers generally return to baseline between six months and one year after infection, our data may not accurately reflect the correlation between reactivity to α -enolase and ASO titer.

In this study, we assessed reactivity to serum anti- α -enolase antibodies in patients with BD and other rheumatologic diseases, including SLE, systemic sclerosis, RA, Takayasu's arteritis, dermatomyositis, and MCTD. Further studies are necessary to determine the diagnostic value of testing for serum anti- α -enolase antibody for treatment of BD and the roles of α -enolase and streptococcal α -enolase in the pathogenesis of BD.

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