

CHINESE MEDICAL SCIENCES JOURNAL

ORIGINAL ARTICLE

Induction of Endothelial Cell Apoptosis by Anti-alpha-enolase Antibody[△]

Hong-bo Yang¹, Wen-jie Zheng^{2*}, Xuan Zhang², and Fu-lin Tang²

¹Department of Endocrinology, ²Department of Rheumatology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100730, China

Key words: anti-alpha-enolase antibody; apoptosis; endothelial cell

Objective To assess the prevalence of anti-alpha-enolase antibody in systemic autoimmune diseases in Chinese patients and its role in endothelial cell apoptosis.

Methods The reactivity of anti-alpha-enolase antibody in a variety of autoimmune disorders in Chinese patients was evaluated by dot blot assay. Endothelial cell apoptosis was investigated by *in vitro* incubation of endothelial cells with IgG purified from anti-alpha-enolase antibody-positive sera, with or without pre-incubation with recombinant alpha-enolase.

Results Anti-alpha-enolase antibody was prevalent in different systemic autoimmune diseases with relatively high reactivity in Chinese patients. *In vitro* incubation of endothelial cells with IgG containing anti-alpha-enolase antibody induced apoptosis in a time- and dose-dependent manner. Apoptosis was partly inhibited by pre-incubation of the endothelial cells with recombinant alpha-enolase.

Conclusions Our data suggest that alpha-enolase is a common auto-antigen recognized by anti-endothelial cell antibodies in connective tissue disease. Interaction between alpha-enolase and its autoantibody plays a role in endothelial cell apoptosis. Changes other than cell killing may contribute to the pathogenesis of endothelial damage and microvascular lesions.

Chin Med Sci J 2011; 26(3):152-157

ANTI-ENDOTHELIAL cell antibody (AECA) has been reported in a variety of clinical settings associated with vasculitis.¹ Although the results obtained in a given disease vary from one study to another, the AECA test shows promise as a se_____

indicator of endothelial cell damage. Our previous work and other reports showed that AECA has a diagnostic role that facilitates monitoring of disease activity and may prove a valuable aid in understanding the common etiopathogenic culitic disorders.²⁻⁴ AECAs

are capable, not only of inducing expression of adhesion molecules⁵ and sustaining leukocyte adhesion to vascular endothelium,⁶ but also of initiating apoptosis of the cells.⁷

⁸ The exposure of phospholipids on endothelium by AECA may be important in triggering the production of anti-

Received for publication January 4, 2011.

*Corresponding author Tel: 86-10-88068177, E-mail: wenjzheng@gmail.com

△Supported by National Natural Science Foundation of China (30400411).

phospholipid antibody in connective tissue diseases.⁹ AECAs represent an extremely heterogeneous family of antibodies reacting with different structures on endothelial cell. The identification of the target antigens and the interactions between those molecules with AECA are critical in further understanding of the mechanisms underlying the effects of AECA on endothelium. Several investigators, including our group, have identified human alpha-enolase as a target antigen of AECA in autoimmune diseases.^{10, 11} In the present study, the prevalence of anti-alpha-enolase antibody in a variety of autoimmune disorders in Chinese patients was investigated and its role in endothelial cell apoptosis was assessed.

PATIENTS AND METHODS

Patients

This study included 169 patients admitted to Peking Union Medical College Hospital during 2001 to 2006 with systemic autoimmune diseases, in whom AECAs reactivity was demonstrated in our previous work: 50 with Behcet's disease (BD), 27 with Takayasu arteritis (TA), 16 with Wegener's granulomatosis (WG), 13 with microscopic polyangiitis (MPA), 10 with Churg-Strauss syndrome (CSS), 23 with systemic lupus erythematosus (SLE), 19 with rheumatoid arthritis (RA), and 11 with primary Sjögren's syndrome (SS). All patients fulfilled the American College of Rheumatology criteria for the respective diseases. The ratio of female to male was 2:1. The age ranged from 16 to 57 years (mean age 42.5 years). Sera from 58 healthy volunteer donors were collected as controls. Informed consent was obtained from patients when a blood sample was collected. Sera were stored at -70°C until used.

Preparation of recombinant alpha-enolase protein

The coding sequence of human alpha-enolase gene was amplified by polymerase chain reaction (PCR) with a pair of primers (5'-ggatccaccatgtctattctcaagatccat-3'; 5'-ccgctcg-agcttggaaggggtttctgaa-3'). The amplified cDNA was subcloned into pET-30a (+) vector (Novagen, Germany). Transformed *Escherichia coli* (DH5alpha, Takara Bio. Inc., Japan) was cultured in lysogeny broth until the absorption density at 600 nm reached 0.45-0.55, as measured by colorimetry. To induce the expression of recombinant protein, 1 mmol/L isopropyl-beta-D-thiogalactopyranoside was added and incubation was continued for an additional 16 hours at 25°C. After centrifugation, phosphate-buffered saline (PBS) with proteinase, in an amount up to 1/100 of the cultured volume, was added. Cells were lysed by sonication on ice. Triton X-100 was then added to a final

concentration of 1%, and the suspension was mixed gently for 30 minutes to facilitate solubilization of the proteins. Recombinant his-tagged protein was purified using kits (Novagen) according to the manufacturer's instructions.

Dot blot assays

One µg of purified proteins was jetted onto a 1 cm×1 cm nitrocellulose membrane (Whatman Schleicher & Schell, Keene, NH, USA). After drying, the membrane was blocked with 1% casein in PBS in an immunoblot reaction tray, and washed several times in PBS. Optimally diluted sera (1:30) were added to each tray containing a membrane in diluent and incubated at 4°C overnight, followed by aspiration and three washes with a Tris buffer (pH 8.0, 10 mmol/L Tris and 0.15% Tween 20). One mL of an HRP-conjugated goat anti-human IgG solution (1:1000 dilution; Promega, USA) was added to each tray. After 1 hour of incubation, each tray was aspirated and washed three times. Immunolabeled dots were visualized by enhanced chemiluminescence detection system (Amersham ECL kit, USA) by exposing to X-ray film (Kodak, Japan). Computer-assisted scanning densitometry (Total Lab, AB.EL Sience-Ware Srl, Rome, Italy) was used to analyze the intensity of the immunoreactive dots. The mean±2SD of the grayscale value of normal controls were taken as the threshold for positivity.

Western blot analysis

IgG fractions in the sera containing AECA against a 47-kDa antigen were purified over a protein G-Sepharose column (Sigma-Aldrich, USA) according to the manufacturer's instructions. The human umbilical vein endothelial cell line, EA.hy926 cells, were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) with 10% fetal bovine serum (Invitrogen). Protein concentration was estimated using the BCA protein assay (Thermo Scientific, Waltham, USA). For SDS-PAGE, lysates were diluted to equal protein concentration in lysis buffer plus 1×NuPage LDS buffer (Invitrogen) supplemented with 2.5% 2-mercaptoethanol. Samples were boiled for 5 minutes, cooled on ice for 1 minute, and vortexed. Equal protein amounts were separated on gradient polyacrlamide gels. Samples were then transferred to Immobilon PVDF membranes (Millipore, USA). Membranes were blocked in 5% milk at room temperature for 1 hour and then immunoblotted with IgG fractions from sera containing AECA against a 47-kDa antigen (1:50) at 4°C overnight. After three washes with Tris buffer (pH 8.0, 10 mmol/L Tris and 0.15% Tween 20), HRP-conjugated goat anti-human IgG solution (1:1000 dilution; Promega) was added. After 1 hour of incubation,

each tray was aspirated and washed three times. Immunolabeled dots were visualized by enhanced chemiluminescence detection system (Amersham ECL kit) by exposing to X-ray film (Kodak).

Assessment of apoptosis

Adherent EA.hy926 cells were incubated with the purified IgG (400 or 800 $\mu\text{g/mL}$) for 72 hours. To further assess whether pre-incubation with recombinant alpha-enolase could prevent EA.hy926 cells from apoptosis induced by anti-alpha-enolase antibody, EA.hy926 cells were incubated with 800 $\mu\text{g/mL}$ of IgG purified from anti-alpha-enolase antibody-positive sera with or without 5 μg of recombinant alpha-enolase for 24 hours. Apoptosis was evaluated by Hoechst staining and flow cytometry.

The cells were then washed, fixed with 4% paraformaldehyde, and stained with Hoechst 33342 (5 g/mL , Beyotime Institute of Biotechnology, China) for 20 minutes at 25°C. The slides were examined by fluorescence microscope and photographed.

The determination of apoptotic EA.hy926 cells was performed using FITC-conjugated annexin V staining and propidium iodide (PI) with a fluorescence microscope and a FACS cytometer (BD Biosciences, USA). Apoptotic cells were the annexin V-positive cells among the PI-negative population. The apoptosis rate was determined by subtracting the number of cells undergoing spontaneous apoptosis.

Statistical analysis

Statistical analysis was carried out with SPSS 14.0. Statistical significance was determined using a two-tailed Student's *t*-test assuming equal variances, and was indicated as $P < 0.01$.

RESULTS

Prevalence of anti-alpha-enolase antibody in systemic autoimmune diseases

Anti-alpha-enolase antibody reactivity was identified in sera from 169 patients with various autoimmune diseases in which AECAs reactivity was demonstrated in our previous work. Dot blot analysis revealed the frequencies of anti-alpha-enolase antibody in patients with systemic autoimmune diseases were as follows: 74.0% in BD, 81.5% in TA, 62.5% in WG, 92.3% in MPA, 80.0% in CSS, 78.3% in SLE, 63.6% in SS, and 78.9% in RA, respectively.

Results of Western blot analysis

Western blot analysis showed the sera from 4 AECA- pos-

itive patients were positive for the 47-kDa endothelial cell antigen (Fig. 1).

Induction of endothelial cell apoptosis by anti-alpha-enolase antibody

In vitro incubation of EA.hy926 cells with IgG obtained from the sera containing anti-alpha-enolase antibody induced apoptosis (Fig. 2) in a time- and dose-dependent manner (Fig. 3). EA.hy926 cells that were annexin V-FITC positive and PI negative were calculated by flow cytometry (Fig. 4). After treatment with 800 $\mu\text{g/mL}$ of IgG purified from anti-alpha-enolase antibody-positive sera from SLE, MPA, MPA and BD patients for 24 hours, the percentage of annexin V positive cells were 21.1%, 17.9%, 16.6%, and 24.2%, respectively. After pre-incubation with alpha-enolase, the percentage of annexin V positive cells decreased to 12.0%, 10.7%, 11.3%, and 10.1%, respectively.

DISCUSSION

Endothelial cells that line the vasculature contribute to the development of inflammatory responses. Because they have long been proved to be a target for immune-mediated assault, it could have been predicted that damage that occurs in the blood vessels is induced by the interaction

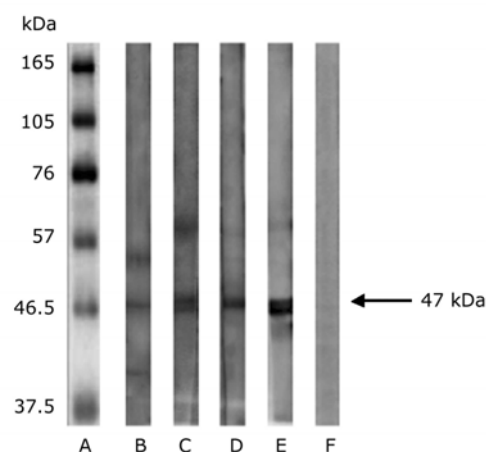


Figure 1. EA.hy926 cells membrane lysate was separated by electrophoresis on 10% sodium dodecyl sulphate-polyacrylamide gel under reducing conditions. Western blot analysis clearly shows bands with strong staining at 47 kDa for 1 patient with systemic lupus erythematosis (SLE, track B), 2 patients with microscopic polyangitis (MPA, track C and D), and 1 patient with Behcet's disease (BD, track E), respectively. No band is visible on the blot exposed to control serum from a normal healthy donor (track F). Track A is the molecular weight marker.

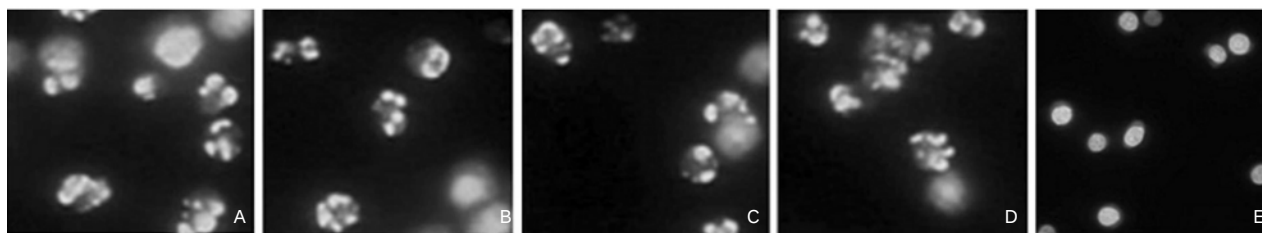


Figure 2. Photomicrograms of apoptosis in EA.hy926 cells after exposure to 800 $\mu\text{g/mL}$ of IgG from anti-alpha-enolase antibody positive patients for 72 hours.

A. IgG from SLE patient; B and C. IgG from MPA patients; D. IgG from BD patient; E. control (treated with IgG from healthy volunteer).

Hoechst 33342 staining shows cells exhibiting apoptotic body and fragmented nuclei.

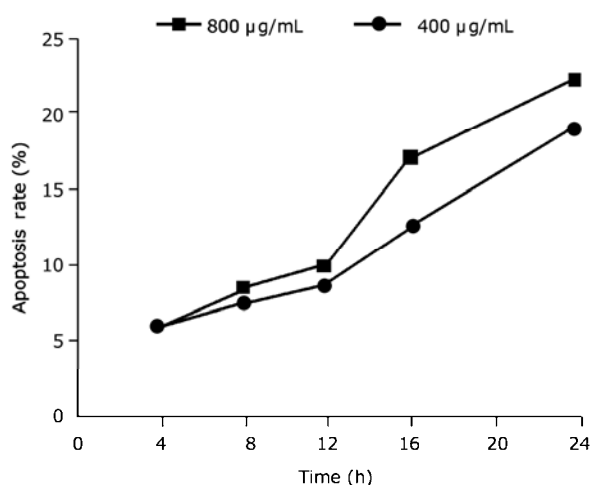


Figure 3. Apoptosis rate of EA.hy926 cells after exposure to IgG from anti-alpha-enolase antibody positive patients for 72 hours analyzed by flow cytometry ($n=4$).

between antibodies and endothelial cells. AECAs are a heterogeneous family of autoantibodies directed against a variety of antigens adhering to endothelial cells. They bind to endothelial antigens and induce endothelial cell damage. According to the previous reports, AECAs are found to be associated with various autoimmune disorders, mainly in the active stage of diseases like SLE,^{12,13} scleroderma,¹⁴ WG,¹⁵ TA,¹⁶ mixed connective tissue disorders (CTD), and so on. The exact mechanism through which AECAs may potentiate endothelial cell activation and vessel wall damage is not yet fully understood. Different pathophysiological effects have been observed in *in vitro* studies, which include direct or indirect cytotoxicity and endothelial cell apoptosis. AECAs positive sera from patients of lupus, WG and SS induce apoptosis of endothelial cells *in vitro*,^{14,15,17} but their target antigens remain unknown.

In our previous work, we identified alpha-enolase to be one of the target antigens recognized by AECAs. Alpha-enolase belongs to a family of cytoplasmic and glycolytic enzymes. Aside from its enzymatic function in the glycolytic pathway, alpha-enolase has been implicated in

numerous diseases, including autoimmune disorders, metastatic cancer, ischemia and bacterial infection. The disease-related role of alpha-enolase is mostly attributed to its immunogenic capacity, DNA-binding ability and plasminogen receptor function, rather than its enzymatic activity. Anti-alpha-enolase antibodies have been detected in kinds of autoimmune diseases, including SLE,¹⁸ RA,¹⁹ BD,²⁰ etc. The prevalence of anti-alpha-enolase antibody in different CTDs varies from report to report. It has been suggested to be specific antibodies of BD²⁰ or early RA.²¹ In 179 randomly selected Chinese patients with CTDs in our cohort, the frequency of anti-alpha-enolase antibody was relatively higher than that reported in previous articles.

Our study suggested that alpha-enolase might be a common auto-antigen recognized by AECAs in CTDs. Though there is strong evidence that cellular immunity plays a major part in the pathogenesis of lots of CTDs, endothelial damage seems to be one of the immunopathological hallmarks in these disorders. Our study further suggested that, changes other than cell killing may contribute to the pathogenesis of the endothelial damage and microvascular lesions. The presence of alpha-enolase on the surface of streptococci may play a crucial role in the induction of autoimmune disease caused by streptococci.²² The prevalence of anti-alpha-enolase antibody indicated a possible infectious trigger of vascular diseases.

In vitro studies suggest that autoantibodies specific for alpha-enolase could play a pathogenic role, either by a cytopathic effect or by interfering with membrane fibrinolytic activity in CTDs.²³ Induction of endothelial cells apoptosis by interactions between anti-alpha-enolase antibodies and its antigens could be prevented by pre-incubation of IgG with recombinant alpha-enolase. Interleukin-6 is not involved in the pathway of endothelial cell apoptosis induced by anti-alpha-enolase antibody (data not shown). Further work is needed to understand the mechanisms thereafter. In addition, induction of endothelial cell apoptosis by the binding of AECAs to heat

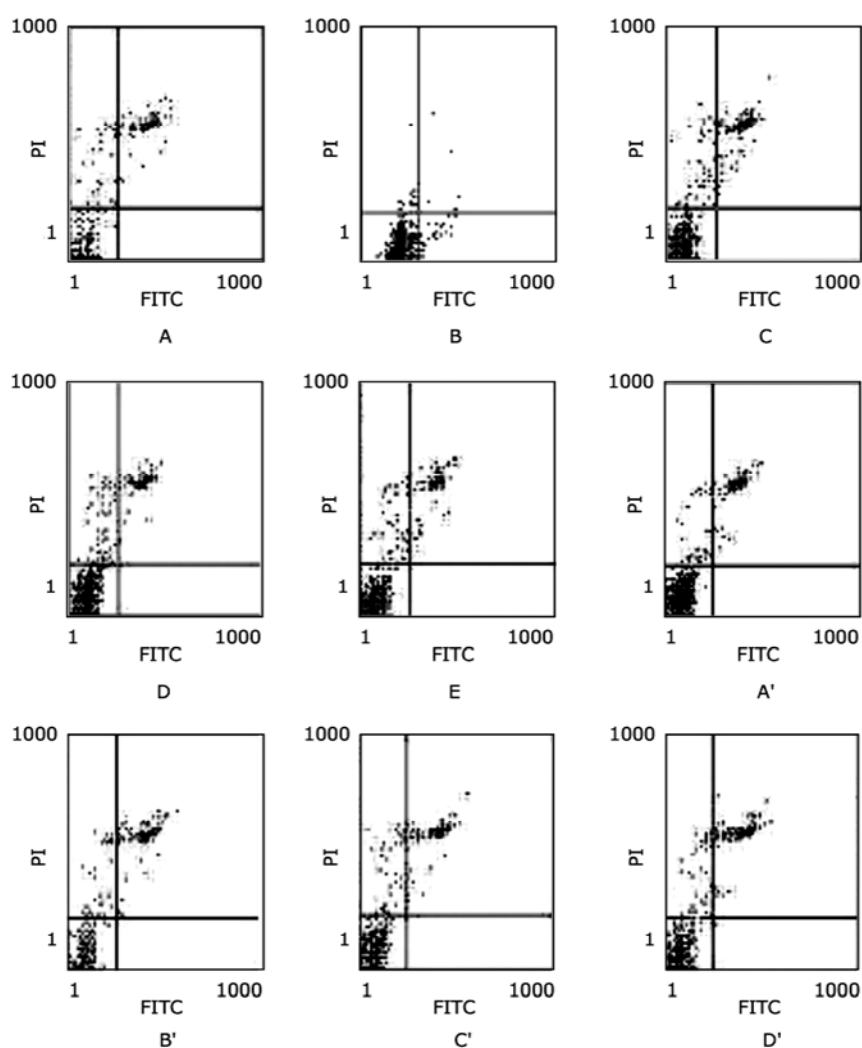


Figure 4. Enumeration of EA.hy926 cells that are Annexin V-FITC positive and PI negative by flow cytometry.

A, A'. IgG from SLE patient; B, B', C, C'. IgG from MPA patients; D, D'. IgG from BD patient; E. control (treated with IgG from healthy volunteer).

EA.hy926 cells in A, B, C, D were incubated with 800 $\mu\text{g/mL}$ of IgG purified from the sera containing anti-alpha-enolase antibody against a 47-kDa antigen; cells in A', B', C', D' were pre-incubated with 5 μg recombinant alpha enolase before adding IgG.

shock protein 60 in vasculitis-associated systemic autoimmune disease has been reported previously. Thus, together with our findings, we believe that endothelial cell damage in CTDs is a complex process and different autoantigens have different roles in the pathologic mechanisms. Identification of autoantigens and their roles will facilitate the diagnosis and treatment of comprehensive autoimmune disorders.

REFERENCES

1. Belizna C, Duijvestijn A, Hamidou M, et al. Antiendothelial cell antibodies in vasculitis and connective tissue disease. *Ann Rheum Dis* 2006; 65:1545-50.
2. Zheng WJ, Zhao Y, Tang FL. Antiendothelial cell antibodies in systemic vasculitis: prevalence and clinical significance. *Chin J Rheumatol* 2005; 9:641-4 (in Chinese).
3. Praprotnik S, Blank M, Meroni PL, et al. Classification of anti-endothelial cell antibodies into antibodies against microvascular and macrovascular endothelial cells: the pathogenic and diagnostic implications. *Arthritis Rheum* 2001; 44:1484-94.
4. Göbel U, Eichhorn J, Kettritz R, et al. Disease activity and autoantibodies to endothelial cells in patients with Wegener's granulomatosis. *Am J Kidney Dis* 1996; 186- 94.
5. Del Papa N, Guidali L, Sironi M, et al. Anti-endothelial cell IgG antibodies from patients with Wegener's granulomatosis bind to human endothelial cells *in vitro* and induce

- adhesion molecule expression and cytokine secretion. *Arthritis Rheum* 1996; 39:758-66.
6. Carvalho D, Savage CO, Black CM, et al. IgG antiendothelial cell autoantibodies from scleroderma patients induce leukocyte adhesion to human vascular endothelial cells *in vitro*. Induction of adhesion molecule expression and involvement of endothelium-derived cytokines. *J Clin Invest* 1996; 97:111-9.
 7. Jamin C, Dugu C, Alard JE, et al. Induction of endothelial cell apoptosis by the binding of anti-endothelial cell antibodies to Hsp60 in vasculitis-associated systemic autoimmune diseases. *Arthritis Rheum* 2005; 52:4028-38.
 8. Sgonc R, Gruschwitz MS, Dietrich H, et al. Endothelial cell apoptosis is a primary pathogenetic event underlying skin lesions in avian and human scleroderma. *J Clin Invest* 1996; 98:785-92.
 9. Bordron A, Dueymes M, Levy Y, et al. The binding of some human antiendothelial cell antibodies induces endothelial cell apoptosis. *J Clin Invest* 1998; 101:2029-35.
 10. Lee KH, Chung HS, Kim HS, et al. Human alpha-enolase from endothelial cells as a target antigen of anti-endothelial cell antibody in Behcet's disease. *Arthritis Rheum* 2003; 48:2025-35.
 11. Zheng WJ, Tang FL, Zhao Y, et al. Prevalence of antiendothelial cell antibody in systemic vasculitis and identification of the target antigen thereof. *Natl Med J Chin* 2005; 85:3272-6 (in Chinese).
 12. Kimura A, Sakurai T, Tanaka Y, et al. Proteomic analysis of autoantibodies in neuropsychiatric systemic lupus erythematosus patient with white matter hyperintensities on brain MRI. *Lupus* 2008; 17:16-20.
 13. Kwok SK, Seo SH, Ju JH, et al. Lupus enteritis: clinical characteristics, risk factor for relapse and association with anti-endothelial cell antibody. *Lupus* 2007; 16:803-9.
 14. Ahmed SS, Tan FK, Arnett FC, et al. Induction of apoptosis and fibrillin 1 expression in human dermal endothelial cells by scleroderma sera containing anti-endothelial cell antibodies. *Arthritis Rheum* 2006; 54:2250-62.
 15. Holmén C, Elsheikh E, Christensson M, et al. Anti endothelial cell autoantibodies selectively activate SAPK/JNK signalling in Wegener's granulomatosis. *J Am Soc Nephrol* 2007; 18:2497-508.
 16. Park MC, Park YB, Jung SY, et al. Anti-endothelial cell antibodies and antiphospholipid antibodies in Takayasu's arteritis: correlations of their titers and isotype distributions with disease activity. *Clin Exp Rheumatol* 2006; 24:S10-6.
 17. van Paassen P, Duijvestijn A, Debrus-Palmans L, et al. Induction of endothelial cell apoptosis by IgG antibodies from SLE patients with nephropathy: a potential role for anti-endothelial cell antibodies. *Ann N Y Acad Sci* 2007; 1108:147-56.
 18. Mosca M, Chimenti D, Pratesi F, et al. Prevalence and clinico-serological correlations of anti-alpha-enolase, anti-C1q, and anti-dsDNA antibodies in patients with systemic lupus erythematosus. *J Rheumatol* 2006; 33: 695-7.
 19. Kinloch A, Tatzer V, Wait R, et al. Identification of citrullinated alpha-enolase as a candidate autoantigen in rheumatoid arthritis. *Arthritis Res Ther* 2005; 7:1421-9.
 20. Yurdakul S, Hamuryudan V, Yazici H. Behcet syndrome. *Curr Opin Rheumatol* 2004; 16:38-42.
 21. Saulot V, Vittecoq O, Charlionet R, et al. Presence of autoantibodies to the glycolytic enzyme alpha-enolase in sera from patients with early rheumatoid arthritis. *Arthritis Rheum* 2002; 46:1196-201.
 22. Fontán PA, Pancholi V, NociariMM, et al. Antibodies to streptococcal surface enolase react with human alpha-enolase: implications in poststreptococcal sequelae. *J Infect Dis* 2000; 182:1712-21.
 23. Moscato S, Pratesi F, Sabbatini A, et al. Surface expression of a glycolytic enzyme, alpha-enolase, recognized by autoantibodies in connective tissue disorders. *Eur J Immunol* 2000; 30:3575-84.