Identification of a target antigen reacting with anti-endothelial cell IgA antibody in Behçet's disease

Sung Bin Cho

Department of Medicine

The Graduate School, Yonsei University

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Directed by Professor Dongsik Bang

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Sung Bin Cho

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This certifies that the Doctoral Dissertation of Sung Bin Cho is approved.

Thesis Supervisor: Prof. Dongsik Bang

Thesis Committee Member: Prof. Kwang Hoon Lee

Thesis Committee Member: Prof. In Hong Choi

Thesis Committee Member: Prof. Seonghyang Sohn

Thesis Committee Member: Prof. Jae Hee Cheon

The Graduate School
Yonsei University

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ABSTRACT

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Sung Bin Cho

Department of Medicine
The Graduate School, Yonsei University

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Behçet's disease (BD) is a chronic, multisystemic vasculitis that theoretically affects all sizes and types of blood vessels. Although pathogenesis remains enigmatic, endothelial cells are believed to be the primary target in this disease, presenting with various symptoms of vasculitis and/or thrombosis. The purpose of this study was to identify human dermal microvascular endothelial cell (HDMEC) antigen, which binds anti-endothelial cell IgA antibodies in BD.

We detected the target protein using western blotting and immunoprecipitation and determined the amino acid sequence of the peptide by liquid chromatography-matrix assisted laser desorption/ionization-tandem time-of-flight analysis (LC-MALDI-TOF/ TOF). We searched for the DNA sequence of the target protein and purified the recombinant target protein by gene cloning. Serum reactivity against the recombinant target protein was analyzed by immunoblotting. Serum reactivity against streptococcal 65-kD heat shock protein (hsp-65) and the recombinant target protein was investigated by enzyme-linked immunosorbent assay (ELISA). In addition, the sera of BD patients and HC as well as cultured *S. sanguis* were used for HDMECs stimulation. Subcellular

fractions of stimulated HDMECs in each group were extracted and immunoblot analyses for the target protein were performed.

The 36-40-kD protein band that was obtained from immunoprecipitation, which was analyzed by LC-MALDI-TOF/TOF, exhibited the amino acid sequences of hnRNP-A2/B1. Reactivity of serum IgA against human recombinant hnRNP-A2/B1 was detected in 25 of 30 BD patients (83.3%), four of 30 systemic lupus erythematosus patients (13.3%), eight of 30 rheumatoid arthritis patients (26.7%), nine of 30 Takayasu's arteritis patients (30%), six of 30 healthy controls (20%), and none of 30 IgA nephropathy patients. Optical densities obtained from ELISAs against the recombinant human hnRNP-A2/B1 in BD were correlated with those against the recombinant streptococcal hsp-65. The hnRNP-A2/B1 was significantly increased in cellular membrane in HDMECs incubated with the sera of BD patients and cultured *S. sanguis* for 12 hours and 24 hours compared with HDMECs incubated with endothelial cell culture media and the sera of healthy controls.

We identified an hnRNP-A2/B1 protein as a target protein of serum anti-endothelial cell IgA antibody in BD patients. We also demonstrated serum IgA reactivity against recombinant human hnRNP-A2/B1 and recombinant streptococcal hsp-65 were correlated.

Key words: Behçet's disease, immunoglobulin A, anti-endothelial cell antibody, hnRNP-A2/B1, *Streptococcus*, heat shock protein 65, subcellular localization

Identification of a target antigen reacting with anti-endothelial cell IgA antibody in Behçet's disease

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I. INTRODUCTION

Behçet's disease (BD) is a chronic, multisystemic vasculitis that theoretically affects all sizes and types of blood vessels[1,2]. Although pathogenesis remains enigmatic, endothelial cells are believed to be the primary target in this disease, presenting with various symptoms of vasculitis and/or thrombosis[2]. Histopathologic features of BD mainly consist of perivascular mononuclear cell infiltration, endothelial cell swelling or necrosis, obliteration of the vessel lumen, and fibrinoid necrosis of vessels[1].

Anti-endothelial cell antibodies (AECA) have been found in serum samples of individuals with various diseases including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), mixed connective tissue disease, systemic sclerosis,

dermatomyositis, Kawasaki disease, and IgA nephropathy (IgAN)[3-7]. AECA has also been detected in serum samples from BD patients and has proven to be associated with disease activity and vasculitis symptoms[3-5]. By using proteomic techniques, including matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry (MS), α-enolase was identified as a target antigen of IgM-type AECA in BD patients[8,9]. However, the pathogenetic role of anti-α-enolase antibody in BD has not been fully elucidated and several studies have shown an association between antibodies against α-enolase and various inflammatory and immune disorders[8-11].

The role of streptococci in BD immunopathogenesis has been demonstrated in several reports[12-15]. BD patients have significantly more *Streptococcus sanguis* (*S. sanguis*) as oral bacterial flora than healthy and other disease controls[12,13]. BD patients show strong delayed cutaneous hypersensitivity reactions as well as oral aphthous ulcerations against streptococcal antigens by skin injection or oral prick with streptococcal antigens[14]. Previously, we reported that sera from BD patients that react with recombinant human α-enolase also cross-react with a *S. sanguis* antigen, which was determined to be streptococcal α-enolase[16,17]. *S. sanguis*, viridans group streptococci, mainly inhabit the mucous membrane of the mouth, throat, colon, and female genital tract. IgA presents in large quantity at mucosal sites and is responsible for mucosal immunity. A previous report revealed that BD patients who were infected with IgA protease-producing *S. sanguis* strains also developed increased IgA titers against these strains as well as against the IgA

protease antigen[18].

The aim of our study was to identify the anti-endothelial cell IgA antibody-binding human dermal microvascular endothelial cell (HDMEC) antigen. We detected a target protein using western blotting and immunoprecipitation and then searched for a similar protein following amino acids sequencing by liquid chromatography-MALDI-tandem time-of-flight (LC-MALDI-TOF/TOF) analysis. We investigated the serum reactivity of the recombinant target protein in BD and other rheumatic diseases. Additionally, serum IgA reactivity against streptococcal 65-kD heat shock protein (hsp-65) was investigated by enzyme-linked immunosorbent assay (ELISA). Also, we investigated the effects of the sera from BD patients and *S. sanguis* on the subcellular localization of the target protein in HDMECs by subcellular fractionation and western blotting.

II. MATERIALS AND METHODS

1. Patients

This study was approved by the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine, Seoul, Korea (4-2009-0744). This study included 30 BD patients who fulfilled the diagnostic criteria of the International Study Group for BD[19]. All patients had active disease, meeting at least two major criteria with or without any number of minor criteria. Sera from 30 healthy volunteer donors, 30 patients with SLE, 30 patients with RA, 30 patients with Takayasu's arteritis (TA), and 30 patients with IgAN, were used as controls. Diagnoses of SLE, RA, and TA were made according to the American College of Rheumatology revised criteria for SLE[20], 1988 revised American Rheumatism Association criteria for classification of RA[21], and American College of Rheumatology 1990 criteria for the classification of TA[22], respectively, by two rheumatologists. The diagnosis of IgAN was made by two nephrologists through biopsy-confirmation accounting the findings of light microscopy, immunofluorescence tests, and electron microscopy. All serum samples were stored at -70˚C.

2. Culture of HDMECs

HDMECs were purchased (Lonza, Basel, Switzerland) and used as dermal microvascular endothelial cells. HDMECs were prepared and treated in a tissue

incubator using endothelial basal medium-2 (Lonza) containing human epidermal growth factor, hydrocortisone, vascular endothelial growth factor, human fibroblast growth factor-B, gentamicin, amphotericin B, R^3-insulin like growth factor-1, ascorbic acid and 2% fetal bovine serum. HDMECs were subcultured serially from passage 3 to 10 at 37℃ in a CO_2 incubator.

3. ELISA with HDMEC antigen

HDMECs were plated in microtiter plates and allowed to grow to confluence over 24 hours. The plates were washed three times with 0.05% phosphate buffered saline solution-Tween 20 (PBST) to block nonspecific binding. Sera (100 μl) from healthy controls (HC) and BD patients was diluted 1:50 in Hanks' balanced salt solution (HBSS; Sigma, St. Louis, MO, USA) with divalent cations and 1% bovine serum albumin (BSA; Sigma) and added to each well of unfixed HDMEC, and the plates were incubated for 1 hour at 37℃. Peroxidase-conjugated goat anti-human IgA antibody (Invitrogen, Carlsbad, CA, USA) was diluted 1:1,000 in HBSS with divalent cations and 1% BSA and added to each well, and the plates were incubated for 1 hour at 37℃. Antibody binding was quantified colorimetrically by adding tetramethylbenzidine (Sigma) as a substrate and the plates were read spectrophotometrically at 450 nm on an ELISA reader (Dynatech, Alexandria, VA, USA).

4. Western blotting using HDMEC antigen

HDMEC antigen in EDTA/BSA was dissolved in buffer (1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 1% Triton X-100) on ice for 30 minutes. After centrifugation at 10,000g for 30 minutes at 4℃, the supernatant was collected and mixed with the same amount of sample buffer. Samples were then loaded into the wells of a 12% polyacrylamide gel. The separated gel was transferred to a nitrocellulose membrane, washed with 0.05% PBST, and incubated overnight at 4℃ in blocking buffer. After a second overnight incubation at 4℃ with gentle agitation in sera from five HC and ten BD patients, diluted 1:500 with PBS, the membrane was washed six times with PBST and then incubated with peroxidase-conjugated goat anti-human IgA diluted 1:10,000 for one hour.

5. Immunoprecipitation

HDMECs were lysed in a RIPA buffer (Tris 50 mM, pH 7.4, NaCl 150 mM, NP40 1%, sodium deoxicholate 0.5%, iodoacetamide 50 mM, PMSF 1mM, soybean trypsin inhibitor 5 mg/ml, and benzamidine 10 mg/ml). Total cell extract (500 μg) was incubated with mixed sera (ten BD patients) for 16 hours. Protein G-Sepharose (Sigma) was pre-incubated with 5 μg of goat anti-human IgA. Then, PBS buffer-washed protein G-Sepharose beads were added and mixed overnight at 4℃. Complexes were sedimented by centrifugation. The immunoprecipitates were suspended in a sample buffer containing 100 mM dithiothreitol and 4M urea and resolved by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel

electrophoresis (PAGE).

6. In-gel digestion and LC-MALDI-TOF/TOF analysis

The excised gel pieces were destained by reduction with a solution of 30 mM potassium ferricyanide/100 mM sodium thiosulfate and then washed with water. The gel pieces were incubated with 0.2M NH_4HCO_3 for 20 minutes, dehydrated and shrunk by 100% acetonitrile twice, and then dried by vacuum centrifugation. For in-gel digestion with trypsin, the gel pieces were rehydrated in the digestion buffer containing 0.05M NH_4HCO_3 and 10 ng/μl of modified porcine trypsin (Promega, Madison, WI, USA) at 4℃ for 30-45 minutes. The excess supernatant was removed and the gel pieces were covered with 30 μl of the 0.05M NH_4HCO_3 buffer. Digestion was performed overnight at 37℃. After in-gel tryptic digestion, tryptic peptides were extracted from the gel particles.

The LC-MALDI-TOF/TOF experiment was carried out using a capillary LC equipped with a Q-TOF Ultima™ mass spectrometer (Waters, Milford, MA, USA). Peptide ions were detected in the data-dependent analysis mode with an MS precursor scan (200-1800 amu) followed by three data-dependent MS-MS scans. For data analysis, the collected raw MS/MS spectra were analyzed with the Mascot Search program (http://www.matrixscience.com/) using both the Swiss-Prot and NCBI human databases[23]. The mass tolerance used for acceptance was 1.0 amu for both molar masses of the precursor peptide and peptide fragment ions. For screening the search data, only peptides yielding larger than a minimum

Mascot score of 30 were accepted as having extensive homology.

7. Construction of expression vectors

The protein-coding regions of human heterogeneous nuclear ribonucleoprotein (hnRNP)-A2/B1 were amplified by PCR using the 5'-oligonucleotide primer CGAGCCTCGAGATGGAGAAAACTTTAGAAACTG containing the underlined *Xho*I restriction site and the 3'-oligonucleotide primers CGGAATTC TCAGTATCGGCTCCTCCCACCATAACCCCCAC containing the underlined *Eco*RI restriction site. Polymerase chain reaction amplification products were gel purified, digested with the appropriate restriction enzymes and cloned into the pRSET bacterial expression vector (Invitrogen). All constructs were confirmed by DNA sequencing.

The protein-coding regions of streptococcal hsp-65 were amplified by PCR using the 5'-oligonucleotide primer CGAGCCTCGAGATGGCAAAAGATATTA AATT, containing the underlined *Xho*I restriction site, and the 3'-oligonucleotide primers CGGAATTCCTACATCATACCGCCCATCATGC, containing the underlined *Eco*RI restriction site. Polymerase chain reaction amplification products were gel purified, digested with the appropriate restriction enzymes and cloned into the pRSET bacterial expression vector (Invitrogen). All constructs were confirmed by DNA sequencing.

8. Bacterial expression and purification

Human hnRNP-A2/B1 and streptococcal hsp-65 were each overexpressed in *Escherichia coli* (*E. coli*) BL21, followed by purification of recombinant proteins to apparent homogeneity using Ni-NTA resin, according to the manufacturer's instructions (Sigma). Protein concentrations were determined with the BCA assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's recommendations, using BSA as a protein standard. Purified recombinant human hnRNP-A2/B1 was confirmed by western blotting using mouse anti-human hnRNP-A2/B1 monoclonal antibody (ab6102; abcam, Cambridge, UK). Purified recombinant streptococcal hsp-65 was confirmed by DNA sequencing. Protein samples were stored at -30℃ until use.

9. Western blotting using recombinant human hnRNP-A2/B1

Purified recombinant human hnRNP-A2/B1 (1 μg) was suspended in sample buffer. The samples were loaded onto a 12% polyacrylamide gel and subjected to electrophoresis at 100V. The membrane was incubated with gentle agitation for overnight at 4℃ with serum samples from HC or patients with BD, SLE, RA, TA, and IgAN, diluted 1:500 with a primary antibody dilution buffer (1% non-fat skim milk, 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20). The membrane was washed six times with PBST and incubated at room temperature for one hour with peroxidase-conjugated goat anti-human IgA antibody diluted 1:10,000 in blocking buffer (1% non-fat dry milk, 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20) and visualized by enhanced chemiluminescence.

10. ELISA using recombinant proteins

Recombinant human hnRNP-A2/B1 and streptococcal hsp-65 were coated overnight at 4°C with 100 μl of each antigen solution in carbonate-bicarbonate buffer containing 2 μg/ml of proteins at pH 9.6. Sera (100 μl) from 30 HC, 30 BD patients, 30 SLE patients, 30 RA patients, 30 TA patients, and 30 IgAN patients were diluted 1:500 in HBSS with divalent cations and 1% BSA, and added to each well of recombinant human hnRNP-A2/B1 and streptococcal hsp-65, respectively. Peroxidase-conjugated goat anti-human IgA antibody was diluted 1:10,000 in HBSS with divalent cations and 1% BSA, and added to each well of recombinant human hnRNP-A2/B1 and streptococcal hsp-65.

11. Bacterial strains and cell stimulation

Cultured *S. sanguis* (American Type Culture Collection (ATCC) 42927) was used for HDMECs stimulation. The microorganisms were grown in tryptic soy broth (TSB; Sigma) with agitation at 37℃ until an optical density at 600 nm (OD600) of 1.0 was reached. Then 1 ml of this culture was combined with 9 ml of TSB and incubated overnight in 75 cm^2 flasks (BD Bioscience, San Diego, CA, USA) at 37℃ without agitation and then, centrifuged at 800 X g for 10 minutes at 4℃. Pellets were washed twice with sterile PBS (pH 7.2) and resuspended in supplemented EBM-2 without antibiotics to obtain bacterial suspensions containing 1 X 10^6 colony-forming units (CFU)/ml and 1 X 10^7 CFU/ml of *S.*

sanguis. HDMECs in 6-well plates, each plate contained 2.5 X 10^5 HDMECs, were incubated for 2 hours in the absence of antibiotics. Next, after 4 hours of co-culturing HDMECs with 2.5 ml of each bacterial suspension, the plates were washed three times with supplemented EBM-2 without antibiotics to remove extracellular microorganisms. Three milliliter of supplemented EBM-2 containing gentamicin and penicillin G was added to the plates and incubated for 3 hours at 37℃ in a 5% CO_2 incubator. Then, the plates were washed three times with supplemented EBM-2 without antibiotics and additional incubation was performed.

Each sera of BD patient and healthy control was diluted 1:50 in supplemented EBM-2 without 2% FBS to stimulate HDMECs for 6 hours, 12 hours, and 24 hours, respectively. HDMECs cultured with supplemented EBM-2 were used as control.

12. Methylthiazoletetrazolium (MTT) assay

To examine the cytotoxicity of each stimulatory condition, HDMECs were cultured in 96-well culture plates, supplemented EBM-2 with 0.5 mg/ml of MTT was added to each well after experimental periods and incubated for 4 hours at 37℃ in humidified 5% CO_2 incubator. Dimethyl sulphoxide (Sigma) was added to the wells, which were further incubated overnight at 37℃. OD was measured with a microplate reader (SpectraMax 340, Molecular Devices, Sunnyvale, CA) at a wavelength of 562 nm. The OD of the HDMECs cultured with supplemented

EBM-2 was designated a relative value of 100. The experiments were performed in triplicate.

13. Subcellular fractionation and immunoblotting

Incubated HDMECs in each group at 6 hours, 12 hours, and 24 hours were harvested with trypsin-EDTA and washed with ice-cold PBS. Membrane, cytoplasm, and nuclear fractions were extracted using the Subcellular Protein Fractionation Kit (Pierce, Rockford, IL, USA), and according to the manufacturer's instructions. Briefly, after adding Cytoplasmic Extraction Buffer (Pierce) to the cell pellet, the tube was incubated at 4℃ for 10 minutes with gentle mixing then centrifuged at 500 g for 5 minutes. Immediately the supernatant, cytoplasmic extract, was transferred to a clean pre-chilled tube on ice. Membrane proteins were extracted by adding ice-cold Membrane Extraction Buffer (Pierce) containing protease inhibitors to the pellet, vortexing and incubating at 4℃ for 10 minutes with gentle mixing. After centrifugation, the supernatant, membrane extract, was collected. Nuclear proteins were extracted with Nuclear Extraction Buffer (Pierce).

The membranes were probed with the following antibodies: mouse anti-human hnRNP-A2/B1 monoclonal antibody (abcam), mouse monoclonal antibody to tubulin (abcam) as a cytoplasmic marker, mouse monoclonal antibody to pan-cadherin (abcam) as a plasma membrane marker, and mouse monoclonal antibody to histone 3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a

nuclear marker. Signals were revealed by enhanced chemiluminescence.

14. Direct immunofluorescence

Oral mucosal tissues, obtained from HC, as well as biopsy specimens of oral ulceration and genital ulceration, obtained from BD patients, were embedded in OCT compound (Tissue-Tek, Sakura Finetek, Torrance, CA, USA), and stored at -80°C. Sections of 5 μm thickness were then cut and air-dried for 1 hour at room temperature prior to fixation in ice-cold acetone. The samples were then incubated in 1% BSA at room temperature for 2 hours and mouse anti-human hnRNP-A2/B1 monoclonal antibody at 4℃ for 18 hours, before being washed three times with PBST. Next, the samples were incubated in the first secondary antibody (Alexa 594 donkey antimouse IgG, abcam) for 1 hour at room temperature and washed another three times with PBST in the dark. The sections were then incubated in 1% BSA for 30 minutes at room temperature in the dark to block any unspecific antibody binding. Finally, the samples were incubated in a second secondary antibody (FITC goat antihuman IgA, Sigma) for 1 hour at room temperature in the dark. The samples were counterstained with 4'-6-diamidino-2-phenylindole (DAPI).

15. Statistical analysis

One-way analyses of variance (ANOVA) with Bonferroni's *post hoc* test were used to analyze the differences in serum reactivity against recombinant human

hnRNP-A2/B1 depending on each disease as well as to analyze the differences in the level of hnRNP-A2/B1 depending on each subcellular fraction of each group. Pearson's correlations were used to examine the significant associations among serum IgA reactivities against recombinant human hnRNP-A2/B1 and streptococcal hsp-65. Chi-square tests, Fisher's exact tests, and Student's *t*-tests were applied to assess differences in the clinical features of BD patients with positive and negative reactivity for recombinant human hnRNP-A2/B1. All analyses were performed using the Statistical Package for the Social Sciences version 18.0 (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant when the *P*-value was less than 0.05.

III. RESULTS

1. Identification of a target protein using HDMEC

ELISA of extracts of HDMECs with serum samples from 30 BD patients was performed, followed by western blotting of extracts of HDMECs with selected serum samples from ten BD patients presenting relatively high optical density values on ELISA. A band of HDMEC protein between 36-kD and 40-kD was detected in all ten patients (Figure 1A) but not in HC or in patients with other rheumatic diseases. Immunoprecipitation also revealed a 36-40-kD protein band of HDMEC antigen reacting with IgA antibodies from BD patients (Figure 1B).

The 36-40-kD protein band obtained by immunoprecipitation was excised from a polyacrylamide gel and digested with trypsin; the resulting peptide fragments were analyzed by LC-MALDI-TOF/TOF, and a peptide fingerprint was obtained. Using the NCBI scanning algorithm, the protein band showed the amino acid sequences of hnRNP-A2/B1 isoform B1 (estimated molecular weight (M_r)/p*I*, 37464/8.97; NCBI accession number, gi| 14043072; Swiss-Prot accession number, NP_112533; Mascot score, 56; sequence coverage, 4%).

2. Expression and purification of recombinant human hnRNP-A2/B1

Human hnRNP-A2/B1 was overexpressed in *E. coli* BL21 followed by purification of recombinant proteins to apparent homogeneity using Ni-NTA resin. The purified proteins were analyzed on a 10% SDS-PAGE at various steps during

the procedure, and the resulting protein bands were stained with Coomassie brilliant blue R250 (Figure 2A). Purified recombinant human hnRNP-A2/B1 was confirmed by western blotting using mouse anti-human hnRNP-A2/B1 monoclonal antibody (Figure 2B).

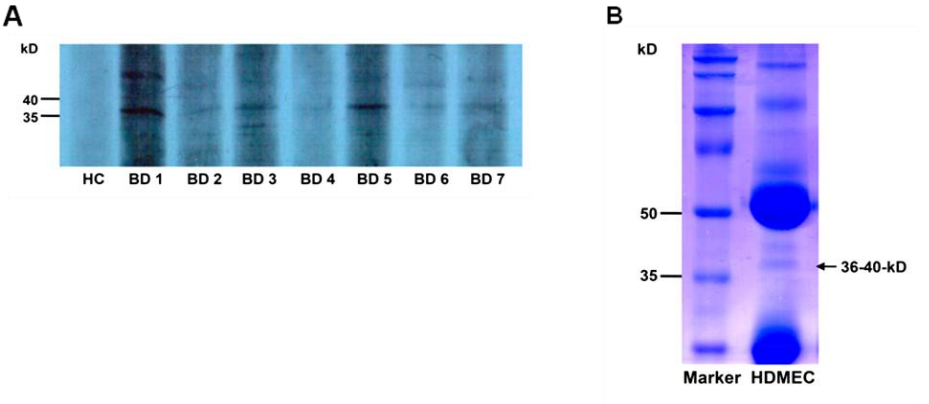

Figure 1. Western blotting and immunoprecipitation using HDMEC antigen. **A,** Western blotting using extracts of HDMECs with selected serum samples of ten BD patients presented a band of HDMEC protein between 36-kD and 40-kD in all ten patients but not in HC or patients with other rheumatic diseases. **B,** Immunoprecipitation was performed to identify the anti-endothelial cell IgA antibody-binding antigen and revealed a 36-40-kD protein band of HDMEC antigen reacting with IgA antibodies of BD patients.

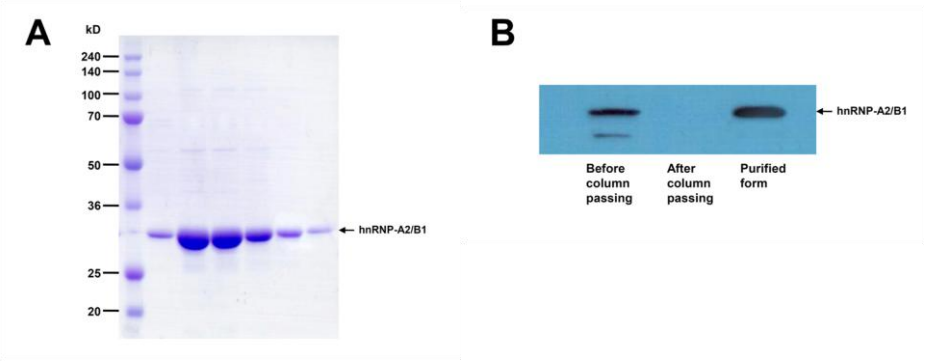

Figure 2. Expression and purification of recombinant human hnRNP-A2/B1. **A,** SDS-PAGE analysis of the purified hnRNP-A2/B1. The purified proteins were analyzed by 10% SDS-PAGE and the protein bands were stained with Coomassie Brilliant Blue R250. **B,** Western blot analysis was performed to confirm the expression of the recombinant hnRNP-A2/B1 in *E. coli* BL21 (DE3) strain.

3. Reactivity of BD sera with recombinant human hnRNP-A2/B1

Western blotting with recombinant human hnRNP-A2/B1 was performed to evaluate serum reactivity using sera from patients with BD, SLE, RA, TA, IgAN, and HC. Reactivity of serum IgA against human recombinant hnRNP-A2/B1 was detected in 25 of 30 BD patients (83.3%), four of 30 SLE patients (13.3%), eight of 30 RA patients (26.7%), nine of 30 TA patients (30%), and six of 30 HC (20%) by western blot, whereas none of 30 IgAN patients presented reactivity against recombinant human hnRNP-A2/B1 (Figure 3). Reactivity of serum IgA against human recombinant hnRNP-A2/B1 was significantly higher compared with those

of HC, SLE, RA, TA, and IgAN patients ($P < 0.0001$, respectively). There were no statistically significant differences in serum IgA reactivity in SLE, RA, TA, or IgAN patients compared with HC ($P > 0.05$, respectively).

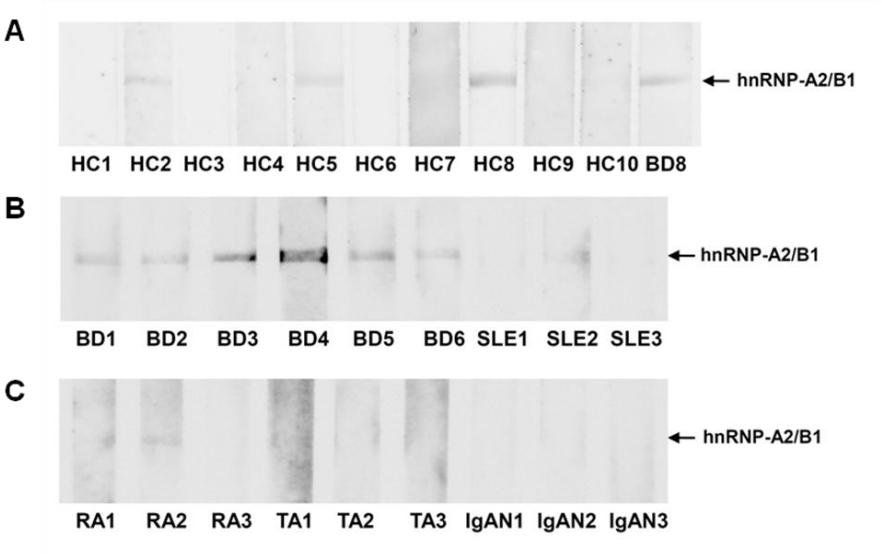

Figure 3. Western blotting using recombinant human hnRNP-A2/B1. Western blotting using recombinant human hnRNP-A2/B1 was performed with sera from patients with BD, SLE, RA, TA, IgAN, and from HC. Reactivity of serum IgA against human recombinant hnRNP-A2/B1 was detected in 83.3% of BD patients (**A** and **B**), 13.3% of SLE patients (**B**), 26.7% of RA patients (**C**), 30% of TA patients (**C**), and 20% of HC (**A**) on western blots, whereas none of 30 IgAN patients presented reactivity against recombinant human hnRNP-A2/B1 (**C**).

4. Correlation of ELISA assay using recombinant proteins

Mean optical density obtained from ELISAs against recombinant human hnRNP-A2/B1 and streptococcal hsp-65 is summarized in Table 1. Optical densities obtained from ELISAs against recombinant human hnRNP-A2/B1 were significantly higher in BD patients ($P < 0.0001$) and TA patients ($P < 0.0001$), but not in SLE patients, RA patients, and IgAN patients, compared with the optical densities in HC. In addition, only BD patients presented significantly higher optical densities against recombinant streptococcal hsp-65 compared with other disease controls and HC ($P = 0.001$).

Optical densities against the recombinant human hnRNP-A2/B1 were correlated with those against the recombinant streptococcal hsp-65 in HC ($r = 0.756$, $P < 0.0001$), BD ($r = 0.774$, $P < 0.0001$), SLE ($r = 0.781$, $P < 0.0001$), and TA ($r = 0.388$, $P = 0.034$) patients, but not in RA and IgAN patients (Figure 4).

Table 1. The mean optical densities of ELISA for autoantibodies against recombinant proteins.

Group	Mean optical density	
	Human hnRNP-A2/B1	Streptococcal hsp-65
HC (N=30)	0.187±0.080	0.133±0.063
BD patients (N=30)	0.374±0.245*	0.256±0.170*
SLE patients (N=30)	0.288±0.131	0.154±0.079
RA patients (N=30)	0.168±0.132	0.139±0.106
TA patients (N=30)	0.370±0.175*	0.137±0.071
IgAN patients (N=30)	0.224±0.099	0.166±0.157

Mean optical density is presented as mean±SD. *$P < 0.001$.

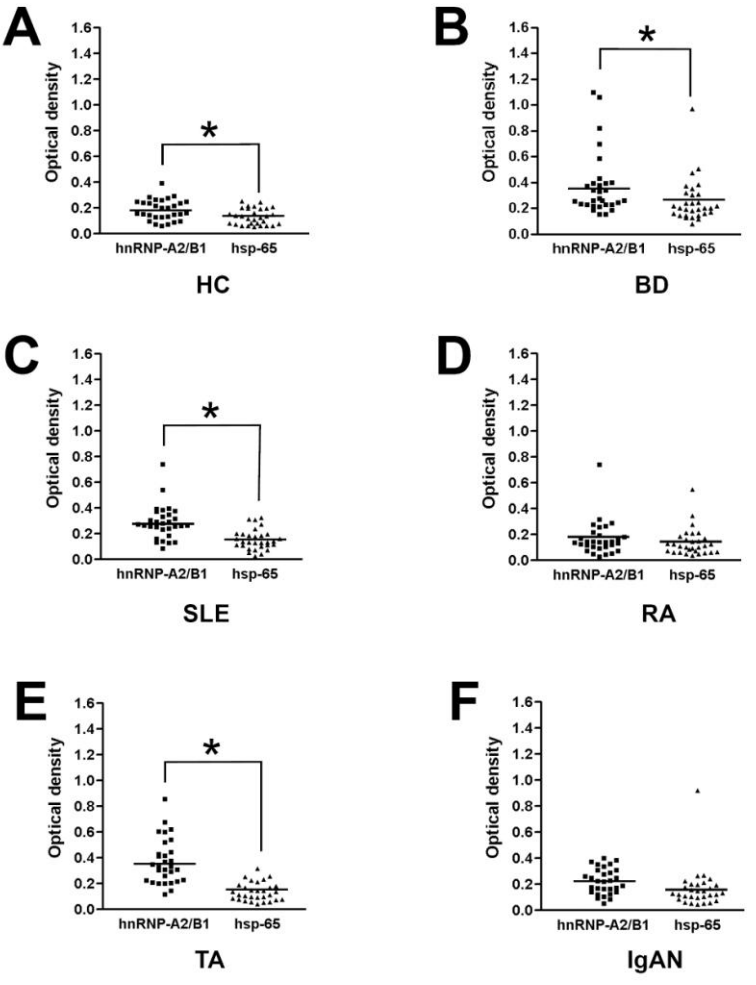

Figure 4. Correlation of ELISA using recombinant proteins. Optical densities obtained from ELISAs of sera against recombinant human hnRNP-A2/B1 were significantly correlated with those against recombinant streptococcal hsp-65 in HC (**A**; $r = 0.756$, $^{*}P < 0.0001$), BD (**B**; $r = 0.774$, $^{*}P < 0.0001$), SLE (**C**; $r = 0.781$, $^{*}P < 0.0001$), and TA (**E**; $r = 0.388$, $^{*}P = 0.034$) patients, but not in RA (**D**) or IgAN (**F**) patients.

5. Clinical significance of anti-hnRNP-A2/B1 IgA antibodies in BD patients

Among the 30 BD patients, 25 patients (eight males and 17 females, mean age 39.8±12.1) showed positive reactivity with recombinant human hnRNP-A2/B1 and five patients (one male and four females, mean age 46±6.1) showed negative reactivity. In BD patients with positive reactivity for recombinant human hnRNP-A2/B1, the following symptoms were observed in descending order of frequency: recurrent oral ulcers in 25 patients (100%), genital ulcers in 21 (84%), skin lesions in 21 (84%), ocular involvement in 16 (64%), articular involvement in nine (36%), gastrointestinal lesions in four (16%), vascular involvement in two (8%), central nervous system involvement in one (4%), and epididymitis in one (4%). A positive HLA-B51 test was noted in nine patients (36%) of the 25 patients with positive anti-hnRNP-A2/B1 IgA antibody.

In BD patients with negative reactivity for recombinant human hnRNP-A2/B1, BD-related symptoms were observed as follows: recurrent oral ulcers and genital ulcers in all five patients (100%), skin lesions in four (80%), articular involvement in four (80%), ocular involvement in two (40%), and gastrointestinal lesions in two (40%). Vascular involvement, central nervous system involvement, and epididymitis were not observed in any of the patients. A positive HLA-B51 test was noted in one patient (20%) of five patients with negative anti-hnRNP-A2/B1 IgA antibodies.

There were no significant differences in any clinical features of BD between the anti-hnRNP-A2/B1 antibody positive and negative groups. Furthermore, the

results of laboratory tests, including full blood count, blood glucose, renal and liver function tests, HLA-B51 genotyping, erythrocyte sedimentation rate, C-reactive protein, anti-streptolysin O titer, rheumatoid factor, antinuclear antibodies, and venereal disease were all similar between the anti-hnRNP-A2/B1 IgA antibody positive and negative groups.

6. Effects of the sera from BD patients on the subcellular localization of hnRNP-A2/B1

The hnRNP-A2/B1 was significantly increased in cellular membrane in HDMECs incubated with each serum of BD patient (BD serum group) at 12 hours and 24 hours compared with HDMECs incubated with endothelial cell culture media (control group; $P = 0.004$ at 12 hours and $P = 0.012$ at 24 hours) and each serum of healthy control (HC serum group; p=0.013 at 12 hours and $P = 0.036$ at 24 hours) (Figure 5A). There were no significant differences among the control group, HC serum group, and BD serum group at 6 hours ($P > 0.05$) and between control group and HC serum group at 12 hours ($P > 0.05$) and 24 hours ($P > 0.05$).

Cytoplasmic hnRNP-A2/B1 was increased in HC serum group and BD serum group for 6 hours ($P = 0.002$ and $P = 0.001$, respectively), 12 hours ($P = 0.004$ and $P = 0.007$, respectively), and 24 hours ($P < 0.0001$ and $P = 0.002$, respectively) compared with control group (Figure 5B). There were no significant differences between HC serum group and BD serum group at 6 hours, 12 hours, and 24 hours.

With 6 hours of incubation, nuclear hnRNP-A2/B1 was decreased in BD serum group compared with HC serum group (P = 0.048), but no significant differences between control group and HC serum group as well as control group and BD serum group (Figure 5C). Otherwise, no significant differences in hnRNP-A2/B1 among control group, HC serum group, and BD serum group at 12 hours and 24 hours.

In cultured media, significantly increased hnRNP-A2/B1 was detected in BD serum group compared to control group (P = 0.009) at 6 hours (Figure 5D). However, there were no significant differences between control group and HC serum group (P > 0.05) as well as HC serum group and BD serum group (P > 0.05) at 6 hours. At 12 hours, although higher levels of hnRNP-A2/B1 were noted in HC serum group and BD serum group, there were no significant differences among control group, HC serum group, and BD serum group (P > 0.05). In addition, hnRNP-A2/B1 was increased in BD serum group compared to control group (P < 0.0001) as well as to HC serum group (P < 0.0001) at 6 hours. Also, HC serum group presented increased hnRNP-A2/B1 compared to control group (P = 0.035).

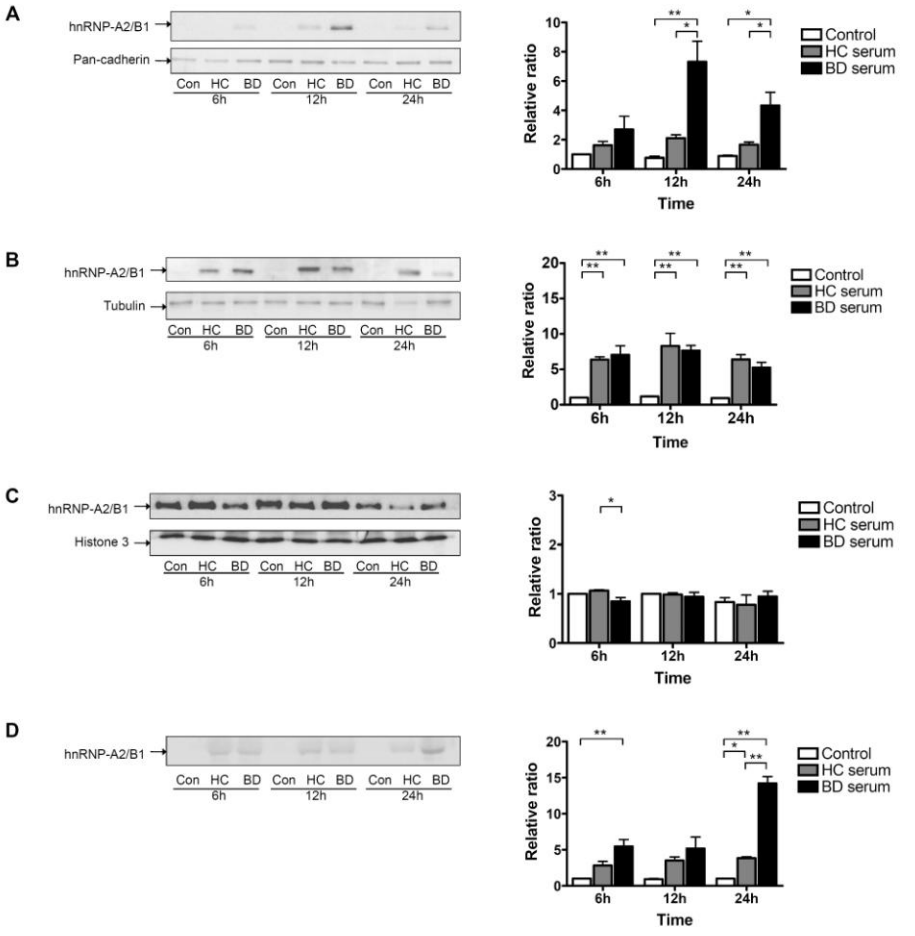

Figure 5. Subcellular localization of hnRNP-A2/B1 in HDMECs incubated with the sera from BD patients. (**A**) The hnRNP-A2/B1 is significantly increased in cellular membrane in endothelial cells incubated with each serum of BD patient at 12 hours and 24 hours. (**B**) Cytoplasmic hnRNP-A2/B1 is increased in healthy control (HC) serum group and BD serum group for 6 hours, 12 hours, and 24 hours compared with control group. (**C**) Nuclear hnRNP-A2/B1 is decreased in BD serum group at 6 hours. (**D**) In cultured media, hnRNP-A2/B1 was detected in BD serum group at 6 hours and 24 hours. Con, control; * $P < 0.05$; ** $P < 0.01$. Data are means±SD.

7. Effects of cultured *S. sanguis* on the subcellular localization of hnRNP-A2/B1

At 6 hours and 12 hours, no significant changes in membranous hnRNP-A2/B1 were observed among control, *S. sanguis* (x10 CFU/HDMEC) group, and *S. sanguis* (x100 CFU/HDMEC) group (Figure 6A). In HDMECs incubated with of *S. sanguis* (x100 CFU/HDMEC), membranous hnRNP-A2/B1 was gradually increased at 6 hours (P >0.05), 12 hours (P > 0.05), and 24 hours (P = 0.015), compared with control group, but not in HDMECs with *S. sanguis* (x10 CFU/HDMEC; P > 0.05).

No significant differences in the level of cytoplasmic hnRNP-A2/B1 were observed at 6 hours, 12 hours, and 24 hours among control group, *S. sanguis* (x10 CFU/HDMEC) group, and *S. sanguis* (x100 CFU/HDMEC) group (Figure 6B).

Nuclear hnRNP-A2/B1 was decreased in *S. sanguis* (x100 CFU/HDMEC) group at 6 hours (P = 0.007) and 12 hours (P = 0.035), but not at 24 hours (P > 0.05), compared with control group (Figure 6C). In *S. sanguis* (x10 CFU/HDMEC) group, nuclear hnRNP-A2/B1 was decreased significantly at 12 hours (P = 0.029) compared with control group and at 24 hours (P = 0.045) compared with *S. sanguis* (x100 CFU/HDMEC) group. However, hnRNP-A2/B1 in cultured media was not detected in all groups at 6 hours, 12 hours, and 24 hours (data not shown).

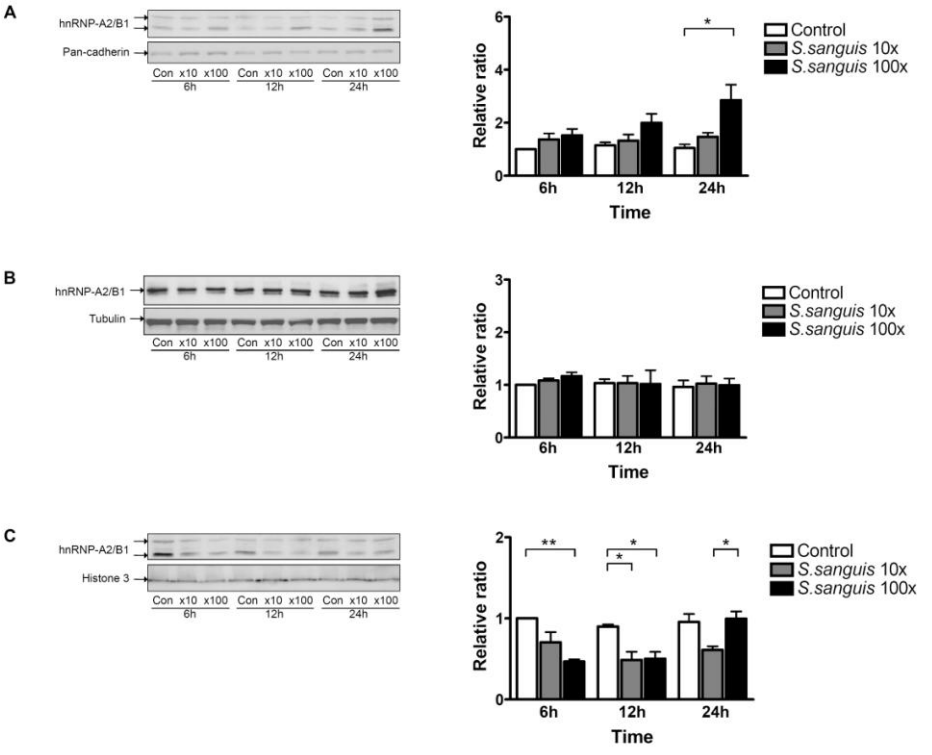

Figure 6. Subcellular localization of hnRNP-A2/B1 in HDMECs incubated with cultured 1 X 10^6 CFU/ml of *Streptotoccus sanguis* (*S. sanguis*; x10) and 1 X 10^7 CFU/ml of *S. sanguis* (x100). (**A**) Membranous hnRNP-A2/B1 is significantly increased in HDMECs incubated with *S. sanguis* (x100) at 24 hours. (**B**) No significant differences in the level of cytoplasmic hnRNP-A2/B1 were observed at 6 hours, 12 hours, and 24 hours. (**C**) Nuclear hnRNP-A2/B1 is decreased between *S. sanguis* (x100) group and control group at 6 hours. Con, control; $^{*}P < 0.05$; $^{**}P < 0.01$. Data are means±SD.

8. Direct immunofluorescence

Specimens obtained from oral ulceration and genital ulceration were found to exhibit immunoreactivity to hnRNP-A2/B1 especially in lower part of the mucosal epithelium and upper dermal mesenchymal and inflammatory cells (Figure 7A, E). On the other hand, oral mucosal specimen of the healthy subject presented immunoreactivity to hnRNP A2/B1 only in part of the surface of the mucosal epithelium (Figure 7I).

Linear and granular IgA deposition was found in both oral ulceration and genital ulceration of BD patients along with the dermoepidermal junctions and perivascular lesions (Figure 7B, F), whereas control mucosal tissue did not present IgA deposition (Figure 7J). In addition, colocalization of serum IgA antibodies, which was deposited in the BD patients' mucosal tissues, and monoclonal antibodies against hnRNP-A2/B1 was detected (Figure 7D, H).

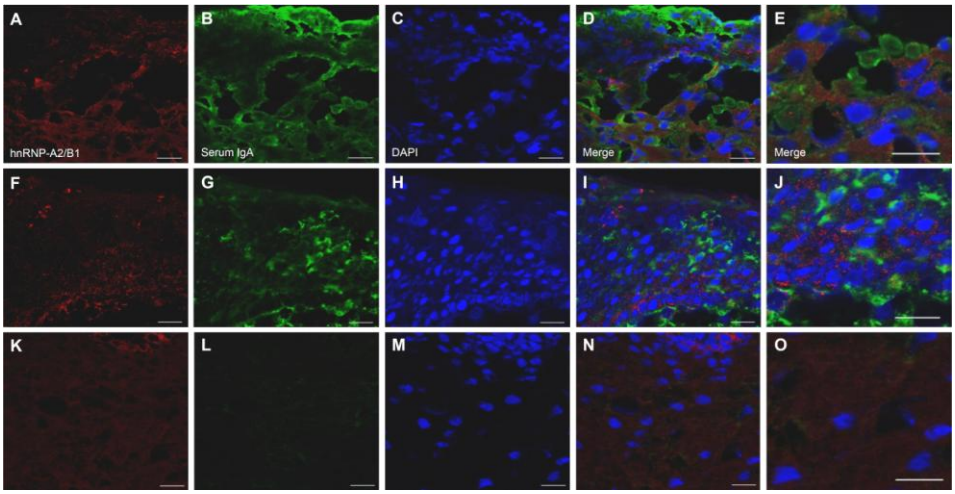

Figure 7. Direct immunofluorescence of mucosal ulcerations in Behçet's disease (BD) patients. Specimens obtained from oral ulceration (**A**-**E**) and genital ulceration (**F**-**J**) of BD patients as well as oral mucosal tissue of healthy subject (**K**-**O**) were processed for confocal immunofluorescence microscopy. Immunoreactivity to hnRNP-A2/B1 was detected in lower part of the mucosal epithelium and upper dermis (**A**, **F**). Linear and granular IgA deposition was found in ulcerative lesions of BD (**B**, **G**) and serum IgA antibodies and monoclonal antibodies against hnRNP-A2/B1 were colocalized (**D**, **E**, **I**, **J**). Bar = 20 μm.

IV. DISCUSSION

Rheumatic autoimmune diseases, including SLE, RA, mixed connective tissue disease, systemic sclerosis, and polymyositis, characteristically present autoantibodies against intracellular antigens[24]. Some specific antinuclear antibodies possess diagnostic significance, such as autoantibodies to double-stranded DNA or Sm antigen in SLE, autoantibodies to topoisomerase in progressive systemic sclerosis, or autoantibodies to tRNA synthetases in poly- or dermatomyositis[24,25]. In contrast to these disorders, pathognomonic autoantibodies to intracellular antigens have not yet been defined in BD.

hnRNPs are RNA binding proteins and can remain with mRNAs until their degradation in the cytoplasm. They participate in the maturation of pre-mRNAs, the nuclear to cytoplasmic transportation of mRNA, and mRNA localization, translation, and stability[26,27]. The hnRNP proteins are also involved in various nuclear events, such as transcriptional regulation, telomere-length maintenance, immunoglobulin gene recombination, splicing, pre-ribosomal-RNA processing, and 3'-end processing[26]. Among the approximately 30 diverse hnRNP proteins, the hnRNP-A/B protein forms a subgroup of closely related proteins. An hnRNP-B1 protein is a splicing variant of hnRNP-A2 and differs from hnRNP-A2 by a 12 amino acids insertion close to the N-terminus[28,29].

hnRNP-A2/B1 overexpression has been described in many cancers, including breast, pancreas, liver, gastrointestinal, and lung cancers[29,30]. Previous

reports suggested that hnRNP-A2/B1 is a marker for early lung cancer[29,31]. Expression of the hnRNP-A2/B1 may play a role in the regulation of fundamental cancer biology, such as migration and aerobic glycolysis[32,33], as well as in epithelial to mesenchymal transition in nonepithelial lung cancer cell lines through the regulation of E-cadherin expression[30]. However, a critical role for overexpressed hnRNP-A2/B1 in the development or progression of cancers has not been fully elucidated.

T cell-driven autoantibody responses in rheumatic diseases, including SLE and mixed connective tissue disease, target a spliceosomal complex, which is mainly composed of small nuclear (sn) RNPs[34]. In addition to snRNPs, some hnRNPs have also been identified as antigenic targets of rheumatic diseases, and serum reactivities against hnRNP-A2/B1 have been described in 30-40% of RA patients, 30-40% of mixed connective tissue disease patients, and 20% of SLE patients[28,35,36]. In the present study, we demonstrated an hnRNP-A2/B1 protein as a target protein of serum anti-endothelial cell IgA antibody in BD patients. Reactivity of serum IgA against human recombinant hnRNP-A2/B1 was detected in 83.3% of BD patients, whereas it was detected in 0-30% of healthy and disease controls. Especially, none of IgAN patients presented reactivity against recombinant human hnRNP-A2/B1 in our study. We think that the different target antigens reacting with circulating IgA in BD and IgAN may contribute to develop different end-organ damage.

We also demonstrated serum IgA reactivity against recombinant human

hnRNP-A2/B1 and recombinant streptococcal hsp-65 were significantly correlated. The hsp-65, derived from oral bacteria including *S. sanguis*, can be detected in the sera and lesions of BD patients and shows considerable homology with peptides of the human hsp-60[15,37,38]. The cross-reactivity of the antibodies in sera from BD patients with *S. sanguis* and some recombinant peptides of hsp-65 derived from *S. sanguis* has been demonstrated[39]. In addition, the serum levels of IgA antibodies to mycobacterial hsp-65, which cross-reacts with selected strains of *S. sanguis*, are increased significantly in BD patients[15,40].

It is widely accepted that certain environmental triggering factors, such as pollutants, ultraviolet light, or infectious agents, play important roles in the pathogenesis of BD in genetically predisposed individuals[1,2,15]. Autoreactive T and B cells are present in the normal mature immune system and can escape from normal immune regulation by the triggering factors resulting immune-mediated end-organ damage. The activation of autoreactive lymphocytes can be influenced by innate immune receptors, such as Toll-like receptors, which primarily recognize pathogen-derived molecular structures but may cross-react with host molecules, particularly nucleic acids. Varying degrees of tissue damage through autoantibodies, direct attack of tissues by autoreactive T cells, and cytokine production may lead to more release of self-antigens[41].

Direct immunofluorescence studies in BD have been demonstrated that IgM and C3 were deposited at the site of inflammatory cell infiltrates in the ulcerative lesions of oral mucosa, whereas IgG and IgA were detected only at

the surface of the ulcer[42]. Previous report demonstrated that the deposition of IgA antibodies on the biopsy specimens was detected in about 25% of the mucosal and skin lesions in BD patients and IgA was deposited exclusively on the perivascular lesions, but not on the dermoepidermal junctions, whereas none of the tissues obtained from the healthy study group presented IgA deposition[43]. However, in the present study, we observed linear and granular IgA deposition in both oral ulceration and genital ulceration of BD patients along with the dermoepidermal junctions and perivascular lesions, whereas control mucosal tissue did not present IgA deposition. We also demonstrated that notable immunoreactivity to hnRNP-A2/B1 was detected in lower part of the mucosal epithelium and upper dermal mesenchymal and inflammatory cells and found the colocalization of serum IgA antibodies and monoclonal antibodies against hnRNP-A2/B1 in the BD patients' mucosal tissues.

Because the hnRNP-A2/B1 is exclusively located in nucleoplasm and cytoplasm, the anti-hnRNP-A2/B1 IgA antibody-mediated immune reaction theoretically can occur after exposing the hnRNP-A2/B1 protein with endothelial cell damage secondary to chronic inflammation. In the present study, subcellular localization analyses revealed that hnRNP-A2/B1 were significantly increased in cytoplasmic membrane of HDMECs, which were incubated with BD patients' serum and cultured *S. sanguis*. We suggest that the changes in the expression and subcellular localization of hnRNP-A2/B1 by the stimulation of the BD sera and cultured *S. sanguis* could have contributed to development of

anti-hnRNP-A2/B1 IgA antibodies and hnRNP-A2/B1 interaction in BD patients.

V. CONCLUSION

We demonstrated that an hnRNP-A2/B1 protein is a target protein of serum anti-endothelial cell IgA antibody in BD patients. We detected serum IgA cross-reactivity between recombinant human hnRNP-A2/B1 and recombinant streptococcal hsp-65. In addition, subcellular localization analyses revealed that hnRNP-A2/B1 were significantly increased in cytoplasmic membrane and cultured media of HDMECs, which were incubated with BD patients' serum and cultured *S. sanguis*. We also demonstrated the colocalization of serum IgA antibodies and monoclonal antibodies against hnRNP-A2/B1 in the BD patients' mucosal tissues. Although we cannot describe a precise pathogenetic role for hnRNP-A2/B1 or for the presence of the anti-hnRNP-A2/B1 antibody, we suggest that an infectious triggering factor, especially *S. sanguis*, activates autoreactive lymphocytes, which recognize streptococcal hsp-65 and then may cross-react with human hnRNP-A2/B1. However, further investigations are necessary to elucidate the roles of hnRNP-A2/B1 and circulating IgA autoantibodies in BD, as well as to determine the diagnostic value of anti-recombinant human hnRNP-A2/B1 IgA antibody tests.

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ABSTRACT (IN KOREAN)

베체트병 환자에서 혈청 면역글로블린 A와 반응하는 인체진피 미세혈관 내피세포 항원의 검출

<지도교수 방동식>

연세대학교 대학원 의학과

조 성 빈

베체트병은 다양한 장기를 침범하는 만성 혈관염에 의한 질환으로 모든 크기와 종류의 혈관에 염증이 발생할 수 있다. 베체트병의 정확한 병인은 아직까지 밝혀지지 않았지만 혈관 내피세포가 이 질환의 일차 표적으로 생각되며 이로 인한 혈관염이나 혈전과 연관된 다양한 증상이 나타난다. 이 연구에서는 베체트병 환자에서 혈청 면역글로블린 A와 반응하는 인체진피 미세혈관 내피세포 항원을 검출하고자 하였다.

이를 위하여 표적항원의 검출을 위해 프로테오믹스 기법을 사용하여 베체트병 환자의 혈청 내 면역글로블린 A와 반응하는 항원을 분석하였다. 검출한 표적항원을 합성하고 이를 이용하여 베체트병 환자 및 다른 류마티스 질환 환자의 혈청 반응도를 측정하였고 베체트병의 병인에 중요한 역할을 하는 것으로 알려진 streptococcal 65-kD heat shock protein (hsp-65)와의 교차반응성을 확인하였다. 또한, 베체트병 환자의 혈청 및 *Streptococcus sanguis* (*S. sanguis*)가 혈관 내피세포 내 표적단백의 발현에 미치는 영향을 알아보았다.

연구 결과 표적항원은 hnRNP-A2/B1인 것으로 나타났고, 이

단백에 대한 혈청의 반응성은 베체트병 환자 30명 중 25명(83.3%), 전신성 홍반성 낭창 환자 30명 중 4명(13.3%), 류마티스 관절염 환자 30명 중 8명(26.7%), 타카야수 동맥염 환자 30명 중 9명(30%), IgA 신병증 환자 30명 중 0명, 정상대조군 30명 중 6명(20%)에서 나타났다. 베체트병 환자의 hnRNP-A2/B1 단백에 대한 혈청 반응도는 streptococcal hsp-65 단백에 대한 혈청 반응도와 연관성이 있는 것으로 관찰되었다. 또한, 혈관 내피세포를 베체트병 환자의 혈청이나 *S. sanguis*로 자극하였을 때 세포질과 핵내에 주로 존재하는 hnRNP-A2/B1 단백이 세포막과 배양액에 나타나는 것을 확인하였다.

이 연구를 통하여 베체트병 환자 혈청 내 면역글로블린 A와 반응하는 혈관 내피세포의 단백은 hnRNP-A2/B1임을 발견하였다. 또한, 베체트병 환자의 hnRNP-A2/B1 단백에 대한 혈청 반응도는 streptococcal hsp-65 단백에 대한 혈청 반응도와 연관성이 있는 것으로 확인하였다.

핵심되는 말: 베체트병, 면역글로블린 A, 항내피세포 항체, hnRNP-A2/B1, *Streptococcus*, heat shock protein 65, 세포내 위치