Vitamin D binding protein plays an important role in the progression of endometriosis

JIN-HEE HWANG[1], TAO WANG[1,3], KYU-SUP LEE[2], JONG-KIL JOO[2] and HONG-GU LEE[1]

[1]Department of Animal Science and Technology, College of Animal Bioscience and Technology, Konkuk University, Seoul 143-701; [2]Department of Obstetrics and Gynecology, School of Medicine, Pusan National University, Yangsan, Gyeongnam 626-770, Republic of Korea; [3]College of Animal Science and Technology, Jilin Agricultural University, Changchun 130118, P.R. China

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Abstract. Endometriosis, characterized by the growth of the endometrial gland and stroma outside the uterine cavity, is a gynecological disorder affecting 6-10% of women of reproductive age. However, the pathogenesis of endometriosis and the molecular mechanisms involved in the progression of this disease remain to be clarified. Therefore, in this study two-dimensional gel electrophoresis (2-DE) combined with mass spectrometry (MS) were applied to explore endometrial proteins with a role in the progression of endometriosis. Expression of global proteins in ectopic endometrial tissue (n=13; endometriosis group) was compared with that of the normal endometrial tissue (n=6; control group). Sixteen differently expressed proteins, including Vitamin D binding protein (DBP), with various functions were primarily identified in the ectopic endometrial tissue. DBP was confirmed to be significantly increased in the ectopic endometrial tissue compared with that in the normal endometrial tissue ($P<0.05$). Results of the present study therefore showed that DBP may play an important role in the progression of endometriosis.

Introduction

Endometriosis, characterized by the growth of the endometrial gland and stroma outside the uterine cavity, is a gynecological disorder affecting women of reproductive age worldwide (1,2). Endometriotic implants arise from retrograde menstruation of endometrial tissue through the fallopian tubes into the peritoneal cavity. Distribution of this ectopic tissue occurs most often within the pelvic peritoneum, but may also occur in the pelvic viscera, rectovaginal spectrum, pleura, abdominal wall, and rarely, the brain (3). Endometriosis affects 6-10% of women of reproductive age and its symptoms include dysmenorrhea, dyspareunia, chronic pelvic pain, irregular uterine bleeding and/or infertility (4).

In endometrial tissue, the ectopic (extrauterine) endometrium (ECE) of women with endometriosis has a similar histological nature compared with its eutopic (intrauterine) endometrium (EUE), although there are a number of different biochemical and functional characteristics (5-7). Previous studies showed that endometrium from women with endometriosis has abnormalities in the structures, proliferation, immune components, adhesion molecules, proteolytic enzymes and inhibitors, steroid and cytokine production and responsiveness, as well as mRNA expression and protein production when compared with endometrium of women without endometriosis (8-11). The association between the etiology and endometriosis remains to be elucidated, however, key pathogenesis might be the characteristics of refluxed endometrium and the peritoneal microenvironment. To gain a better understanding of the factors involved in the onset of endometriosis, the molecular mechanisms of endometriosis should be investigated.

Currently, proteomic analysis is the preferred tool used for understanding this disease as it enables the estimation of a number of proteins in complicated protein mixtures and quantitative distinctions of the alterations in protein profiles (10,12,13). In addition, the combinatorial system of two-dimensional electrophoresis (2-DE) and mass spectrometry (MS) has been extensively applied to scan for differentially expressed proteins. Proteomics and mass spectrometry have been previously used to investigate endometriosis using various sample types including serum, peritoneal fluid, eutopic and ectopic endometrial tissue, and endometrial fluid (14-18).

The aim of this study was to investigate the characteristic of endometriosis on a molecular level by using 2-DE combined with MS. Global proteins in ectopic endometrial tissue with endometriosis were compared with those from the normal endometrial tissue. The present study may provide insight into

Correspondence to: Dr Hong-Gu Lee, Department of Animal Science and Technology, College of Animal Bioscience and Technology, Konkuk University, 120 Neungdong-ro, Gwangjin-gu, Seoul 143-701, Republic of Korea
E-mail: hglee66@konkuk.ac.kr

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the pathogenesis of endometriosis as well as understanding of molecular mechanisms involved in the progression of this disease.

Materials and methods

Subjects. This study was approved by the ethics committee of the Institutional Review Board at the Pusan National University Hospital (PNUH IRB 2010144). Patients and controls were required to sign a consent form prior to participation in the study. Eutopic endometrial tissues were collected from the patients who underwent laparoscopy or laparotomy for myomectomy or benign ovarian cyst other than endometriosis, such as mature cystic teratoma, functional cyst and hemorrhagic cyst (ages 25-48; n=6). Patients presenting with abdominal/pelvic pain, dysmenorrhea and/or subfertility underwent laparoscopy and were histologically diagnosed as endometriosis. These patients (aged 27-40; n=13) comprised the endometriosis group. Samples were collected during the proliferative phase. Biopsy specimens were obtained for to histologically confirm the diagnosis and the extent of the disease was staged according to the revised American Fertility Society (rAFS) classification. The tissue samples were frozen in liquid nitrogen and preserved at -80˚C until required.

Protein sample preparation. Ectopic endometrial tissue samples stored at -80˚C were thawed, weighed and combined with lysis buffer containing 7 M urea, 2 M thiourea, 4% w/v 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM dithiothreitol (DTT), 0.5% (v/v) pharmalyte (pH 3-10NL) and protease inhibitor (GE Healthcare, Piscataway, NJ, USA). The tissues were homogenized and then agitated every 5 min for 1 h, after which the samples were centrifuged at 17,000 rpm for 30 min at 20˚C. The supernatant was quantified by the 2-D Quant kit (GE Healthcare) and stored at -80˚C until required for further analysis.

2-DE and protein identification by electrospray ionization-quadrupole-time of flight/mass spectrometer ESI-Q-TOF/MS. Individual protein samples were divided into the endometriosis and control groups. The electrophoretic separation of these proteins was performed as previously described (19). Briefly, proteins were diluted into isoelectric focusing (IEF) buffer and then 100 μg of proteins were loaded onto the pH 3-10 NL Immobiline DryStrip gels (GE Healthcare; 18 cm). The gels were used to rehydrate the strips at 20˚C for 12 h. IEF was performed with an IEF electrophoresis unit (GE Healthcare) after 12-h rehydration. The focusing conditions started at a linear ramp from 500 to 1000 V for 1 h and a constant voltage of 8000 V for 6 h to give a total voltage of 56,000 Vh. After focusing, each strip was equilibrated for 15 min in an equilibration buffer containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 2% SDS, 30% glycerol and 0.002% (w/v) bromophenol blue. The first equilibration buffer contained 1% DTT and the second 135 mM iodoacetamide (IAA). The Ettan DALT 2-D gel system (GE Healthcare) was used for electrophoresis along the second dimension. The equilibrated strips were inserted into 12% SDS-PAGE gels (18 cm) and the separated gels were stained using a PlusOne Silver staining kit (GE Healthcare). Spot detection, pair matching and normalization were carried out using the ProteomWeaver software 2.2 (Definiens, Munich, Germany). The ratios of the spots with ≥2-fold changes in staining intensity were selected for ESI-Q-TOF/MS analysis. Details of the ESI-Q-TOF/MS analysis have been described in a previous study (20).

Western blot analysis. A total of 11 individual samples in endometriosis group (rAFS II =1, rAFS III =4, rAFS IV =6) and 4 samples from control group were used for western blotting. Tissue protein was separated by 10% SDS-PAGE gel electrophoresis and then transferred for 30 min onto nitrocellulose membranes by using a Trans-blot® SD Semi-dry Transfer cell (Bio-Rad, Hercules, CA, USA). The membranes (Whatman, Dassel, Germany) were immediately placed into a 5% non-fat milk solution and blocked for 1 h at room temperature. They were incubated overnight at 4˚C with vitamin D binding protein (DBP) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and flavin reductase (FR) (Santa Cruz Biotechnology, Inc.) antibody at a dilution of 1:125 and 1:500, respectively, in TBST. The membranes were then washed three times with 1X TBST and incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Thermo scientific, Rockford, IL, USA) and rabbit anti-goat IgG (Abcam, Cambridge, MA, USA) at 1:10,000 and 1:1,000 dilution for 20 min at room temperature. The proteins on the membrane were visualized by an enhanced chemiluminescence system (ECL) detection kit (Surmodics, Eden Prairie, MN, USA) and bands were quantified using Image J 1.43 software (http://rsb.info.nih.gov/ij/download.html). The protein levels were normalized to those of β-actin signal on the same membrane.

Statistical analysis. Data were expressed as means ± SEM. Comparisons of two groups were analyzed by the Student's t-test and those of multiple groups were analyzed by the Tukey HSD Kramer comparison test. JMP 7.0.1 (SAS Institute Inc., Cary, NC, USA) was used for statistical analyses. $P<0.05$ was considered to indicate statistical significance.

Results

Protein identification in ECE tissue from women with endometriosis. A total of 406 spots were detected in the endometriosis group (n=13) and a total of 385 spots were identified in the control group (n=6). Among these proteins, 50 spots were found to be differentially expressed with spot density changes of approximately ≥2-fold. Twenty spots were decreased whereas 15 spots were increased in the endometriosis group compared with those in the control group. Moreover, eight spots were expressed only in the control group and seven spots were expressed only in the endometriosis group (Fig. 1). Sixteen selected spots were compared according to clinical stage (stage II=1, III=4 and IV=8) of endometriosis through 2-DE analysis (Fig. 2). Among these spots, the density of 9 spots gradually increased whereas the density of 7 spots gradually decreased from stage II to IV (Fig. 2). All 16 spots were selected and identified by ESI-Q-TOF/MS (Table I). The densities of three candidate proteins are shown in Fig. 3. The three proteins were statistically increased in the endometriosis group compared with the control group ($P<0.05$). The proteins

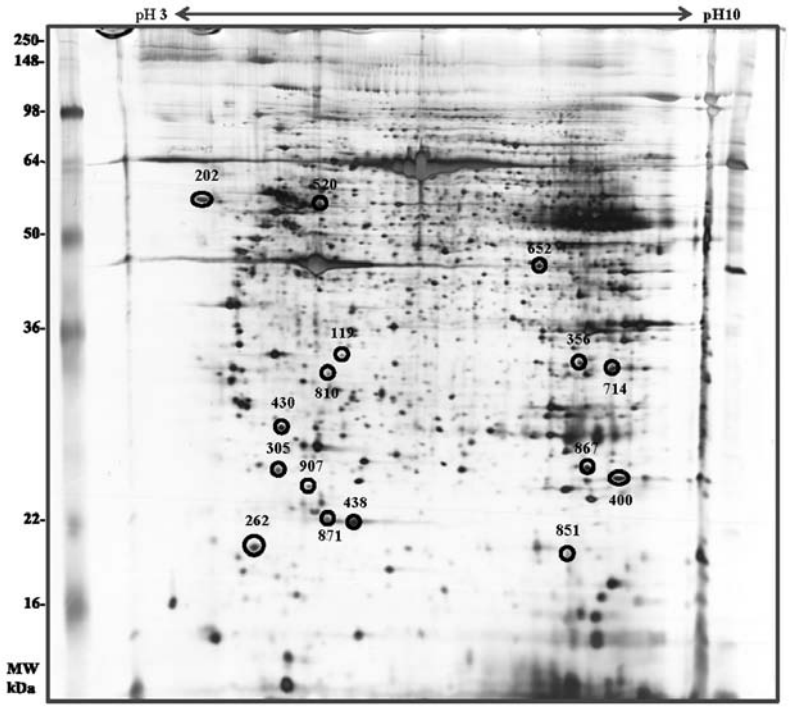

Figure 1. Proteomic analysis of highly expressed proteins in ectopic endometrial tissue from the endometriosis group.

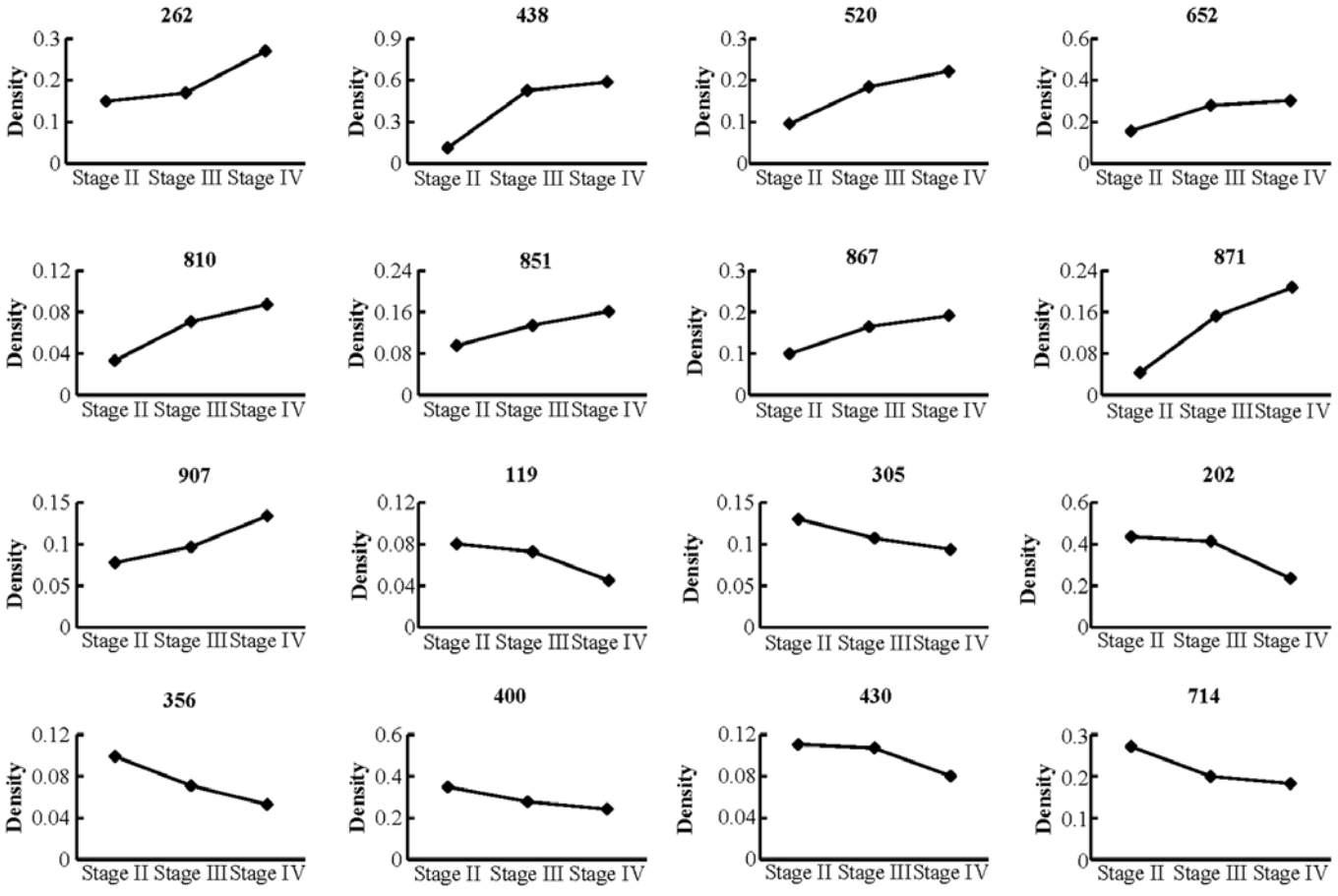

Figure 2. Protein expression patterns in ectopic endometrial tissue according to the rAFS stage of endometriosis. Spots were based on proteins pooled according to the stage (rAFS; stage II=1, stage III=4, stage IV=8). Graphical representation indicates spots showing an increased or decreased expression from stages II to IV. Corresponding proteins are shown in Table I.

Table I. Identification of differentially expressed protein spots in ectopic endometrial tissue by ESI/Q-TOF MS.

Spot no.	Accession no.	Protein name	Score	Mol. wt. (kDa)/pI	Expression	Molecular functions
119	gil74718831	Glyoxalase domain	242	34.8/5.40	Down	Glycolytic enzyme
202	gil62897681	Calreticulin precursor	114	48.1/4.29	Down	Apoptosis regulation
262	gil16418467	Myosin regulatory	6	19.8/7.78	Up	Regulation of both smooth muscle and nonmuscle cell contractile activity via its phosphorylation
305	gil62089188	Lactoylglutathione lyase	55	20.8/5.12	Down	Ion transport
356	gil55664663	Voltage-dependent anion	382	31.6/7.50	Down	Apoptosis regulation
400	gil55959887	Peroxiredoxin-1	453	22.1/8.27	Down	Apoptosis regulation
430	gil36038	Rho GDP-dissociation	272	23.2/5.01	Down	Apoptosis regulation
438	gil48145547	Ferritin light chain	670	20.0/5.50	Up	Cellular homeostasis
520	gil455970	Vitamin D-binding protein	41	53.0/5.40	Up	Carrier vitamin D sterols
652	gil3641398	NADP-dependent isocitrate	79	46.7/6.53	Down	Citric acid cycle enzyme
714	gil119630158	Carbonyl reductase	89	30.4/8.55	Down	NADPH-dependent
851	gil48255905	Transgelin (TAGLN)	158	22.6/8.87	Up	Calcium interactions
867	gil4502419	Flavin reductase (NADPH)	92	22.1/7.13	Up	Oxidoreductase
871	gil48145547	Ferritin light chain (FTL)	446	20.0/5.50	Up	Cellular homeostasis
907	gil5453559	ATP synthase subunit d	15	18.5/5.21	Up	Ion transport

were DBP (520), FR (867) and peroxiredoxin-1 (PRDX1) (400). In addition, the expression of those proteins was investigated according to the clinical stages. The results showed that DBP and FR were statically significant ($P<0.05$) and the two proteins were selected as validation proteins in the subsequent western blotting analysis.

Validation test by western blotting. Results of the western blot analysis revealed that the expression of DBP was significantly increased in the endometriosis group when compared with that in the control group ($P<0.01$) (Fig. 4A and B). This protein also showed an increased pattern in stages II and IV of the endometriosis group compared with the control group (Fig. 4C and D). Another selected candidate protein, FR, exhibited an increase in the endometriosis group compared with the control group, although the difference not significant (Fig. 4).

Discussion

In the present study, 2-DE combined with MS were used to explore proteins that may be involved in the progression of endometriosis. Two proteins identified in the ECE tissue were gradually increased according to disease progression (stage II to IV) in the 2-DE gel image analysis. Spots were identified by MS as DBP and FR. In the subsequent validation test, using western blotting, DBP showed a significantly higher expression level in the endometriosis group compared with the control group. However, no significant difference was found for FR.

DBP is ~58 kDa in size and is genetically related to serum albumin, α-fetoprotein and afamin (21-23). It is a polymorphic serum glycoprotein with lots of functions that include highly specific binding of vitamin D sterols, G-actin, fatty acids and chemotactic agents. It is produced in the liver and is located predominantly in the serum (24-26). It has two large domains (I and II) and a shorter domain at the COOH terminus (domain III) (26). Although it is derived from the plasma (or serum), it is expressed in various tissues and is synthesized in the cell membrane-associated lymphocytes, monocytes and neutrophils (26-28). It is associated with macrophage activation, increases monocyte and neutrophil chemotaxis to C5-derived peptides and plays a role as an actin scavenger protein (29,30). However, the connection between DBP and endometriosis has not been studied in detail, with only a few papers reporting its expression in endometriosis. In previous studies, DBP presented various expression aspects in urine, peritoneal fluid and plasma (31,32). In recent studies, proteomic analysis of the serum and urine showed that the concentration of DBP is elevated in endometriosis patients compared with controls (33,34).

Our results show that the expression of DBP in ECE tissue is increased in patients with endometriosis in accordance with rAFS. Hunt *et al* demonstrated that DBP was synthesized by DBP gene transcription in liver, kidney and brain (in order of abundance) (35). DBP was synthesized in the liver at low levels until 40 weeks after conception and the expression presumed adult levels of 500 μg/ml in serum (36). It was also detected in the lung, heart, stomach, spleen, uterus and brain using PCR of DBP cDNA (35). Results obtained in the present study suggest that DBP transcription occurs actively in ECE and the DBP expression level accelerates with its deterioration into a deeper stage.

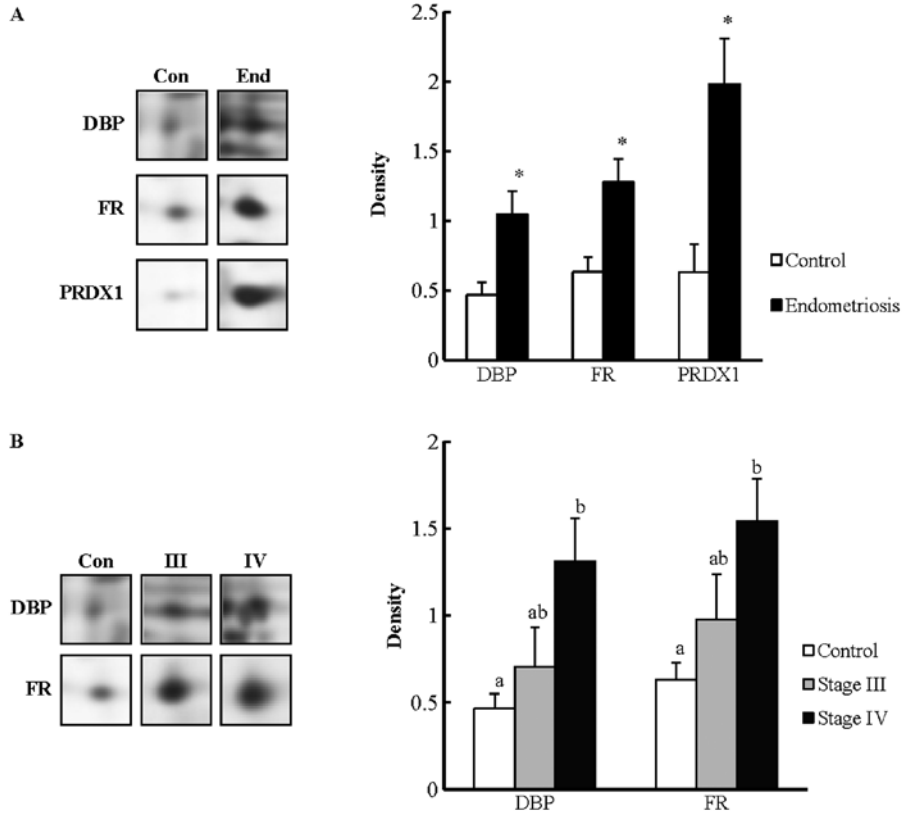

Figure 3. Spot densities of vitamin D binding protein (DBP), flavin reductase (FR) and peroxiredoxin-1 (PRDX1) in ectopic and normal endometrial tissue (EUE). Selection of proteins among those identified by ESI/Q-TOF in individual ectopic endometrial tissue (ECE) samples. Experiments were performed with ECE from 12 different individual samples (con=6, stage III=4, stage IV=8) from endometriosis and control patients. (A) DBP, FR and PRDX1 were observed to be statistically significant in the endometriosis group compared with the control group. (B) DBP and FR showed a significant difference in the server stage (stage III and IV) compared with the control. Values are expressed as the mean ± SEM. *P<0.05 vs. control (Student's t-test) and ^{a,b}P<0.05 vs. control (Tukey's HSD Kramer test).

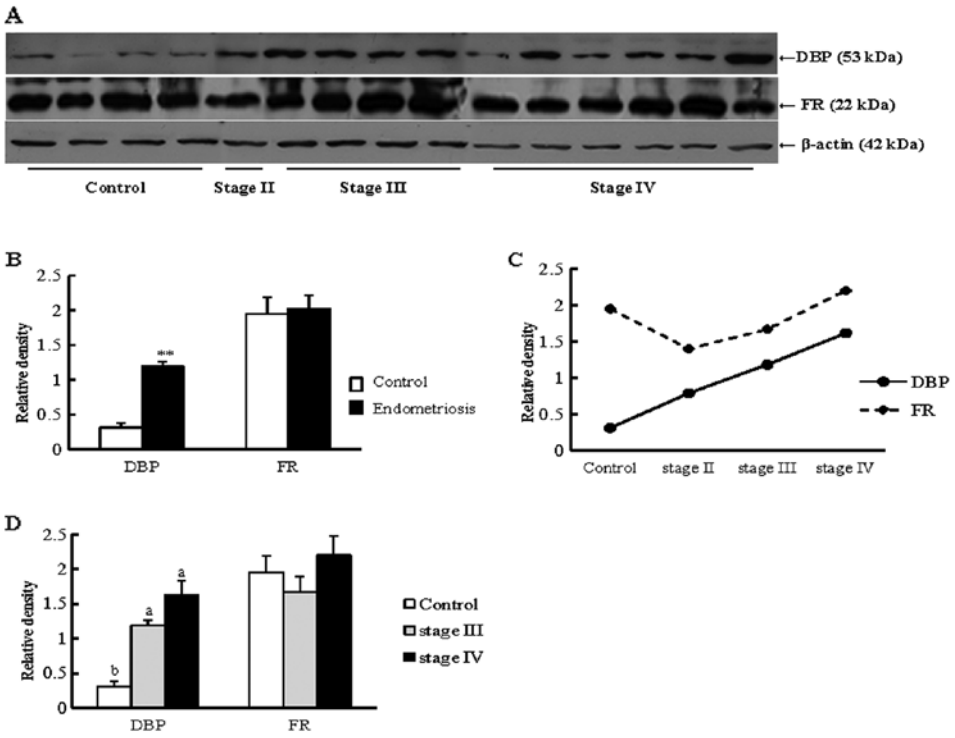

Figure 4. Validation of plasma proteins by western blotting. (A) Western blot analysis of 18 ECE samples probed with anti-vitamin D binding protein (anti-DBP) and anti-flavin reductase (anti-FR) antibodies. (B) The relative densities of proteins in the endometriosis group compared with the control group. (C) Proteins were expressed as a pattern according to clinical stages. (D) Protein expressions were compared with stages III and IV against the control. Values are expressed as the mean ± SEM. **P<0.01 vs. control (Student's t-test) and ^{a,b}P<0.05 vs. control (Tukey's HSD Kramer test).

DBP is known as a chemotactic factor that recruits neutrophils, monocytes and fibroblasts, as it plays an important role in the immune system (28,37,38). DBP is the precursor of macrophage-activating factor (MAF). This activity was confirmed when mouse peritoneal cells were stimulated with lysophosphatidylcholine, ensuring macrophages with increased phagocytic activity (29,39). DBP is converted to its active form, and is partially deglycosylated by β-galactosidase and sialidase activities of the B- and T-cells, producing DBP-MAF (29). It is a powerful activator of macrophage functions and is involved in immune responses. The conversion of DBP to DBP-MAF may be reduced in malignancies by the action of α-N-acetylgalactosaminidase (40). Thus, activation of DBP-MAF suggests that it has a positive effect on the pathogenesis of the disease. Thus, the action of DBP-MAF may also have a similarly good impact on endometriosis in the form of cancer. However, several studies have shown differential DBP-MAF function among DBP genotypes (41-44). DBP has three major allele products (GC1F, GC1S and GC2) (47). Several studies have found that the GC1F form of DBP is correlated with increased chronic obstructive pulmonary disease (COPD) risk for Asians, whereas the GC2 form is associated with reduced COPD in whites (41-43,46,47). In addition to COPD, GC2 was also associated with increased risk to bronchiectasis (41). Another study revealed that DBP genotypes have different glycosylation rates. Borges *et al* showed that the allele products of GC1 were glycosylated at a total rate of 10-30% and those of GC2 were glycosylated at a rate of 1-5% (48). In the endometriosis study, there were related DBP genotypes. Faserl *et al* demonstrated that the DBP allele products in serum were different because of the reduced ability of DBP to convert to DBP-MAF, the essential macrophage activator. According to that study, the form of DBP encoded by the GC1 allele was much more readily converted to DBP-MAF, while that encoded by GC2 was not readily converted, i.e., none of the women in the control group expressed only GC2 allele products, whereas they were expressed in a much higher percentage of women with endometriosis. As stated above, overexpression of the GC2 allele in endometriosis patients may act on the scavenger function of macrophages (33). In view of our study, increased DBP expression in endometriosis patients and stages suggests that GC2 allele products may yield the survival and implantation of ectopic endometrial tissues in the peritoneal cavity or other places.

In conclusion, DBP may be crucial in the progression of endometriosis. However, additional studies are necessary to determine the association between DBP genotypes and the development and progress of endometriosis.

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