Polymorphism in Vitamin D-Binding Protein as a Genetic Risk Factor in the Pathogenesis of Endometriosis

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Context: Previous studies have implicated a deficiency in the inflammatory response in women who develop endometriosis. The specific immunological deficits have not been completely elucidated.

Objective: Our objective was to identify differences in protein expression in serum that might shed light on the pathophysiology of endometriosis.

Design and Setting: This cross-sectional study of women undergoing laparoscopy between 2003 and 2005 took place at a university medical center.

Patients: Patients included consenting women age 18–49 yr undergoing surgery for pain and/or infertility or elective tubal ligation. Women with acute or chronic medical conditions were excluded.

Intervention: Blood was collected preoperatively.

Main Outcome Measure: Proteomic analysis of serum was done using two-dimensional difference gel electrophoresis.

Results: We found 25 protein spots with a significant difference in abundance between women with endometriosis and controls, including acute-phase proteins and complement components. The abundance of vitamin D-binding protein was higher in all endometriosis pools by a factor of approximately 3 compared with the control pool ($P < 0.02$). Analysis of specific allele products using nano-scale liquid chromatography-electrospray ionization-mass spectrometry indicated that it was the GC*2 allele product that was in greater concentration in serum pools, as well as in single validation samples, in women with endometriosis ($P = 0.006$). In contrast to the GC*1 allele product, which is readily converted to a potent macrophage factor (Gc protein-derived macrophage-activating factor), the GC*2 allele product undergoes practically no such conversion.

Conclusions: We speculate that the inability to sufficiently activate macrophages' phagocytotic function in those carrying the GC*2 polymorphism (more prevalent in endometriosis) may allow endometriotic tissues to implant in the peritoneal cavity. Future studies evaluating specific vitamin D-binding protein polymorphisms as a risk factor for endometriosis in larger populations of women are warranted. **(*J Clin Endocrinol Metab* 96: E233–E241, 2011)**

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/jc.2010-1532 Received July 6, 2010. Accepted September 27, 2010.
First Published Online October 27, 2010

Abbreviations: DBP, Vitamin D-binding protein; 2D DIGE, two-dimensional difference gel electrophoresis; GcMAF, Gc protein-derived macrophage-activating factor; IPG, immobilized pH gradient; MS, mass spectrometry; MS/MS, tandem MS; pI, isoelectric point; Xcorr, cross-correlation number.

Endometriosis, the ectopic presence of endometrial glands and stroma is a common disease of reproductive age women. It affects about 10% of all women and upwards of 40% of women with infertility (1). The precise etiology of endometriosis remains unclear.

The most accepted theory regarding the pathophysiology of endometriosis is that it arises from retrograde menstruation, leading to the subsequent adhesion of endometrial tissues in the pelvis (2). In recent years, this theory has been considerably extended and supplemented by studies demonstrating the significance of uterine autotraumatization of basal endometrium caused by hyperperistalsis activating basic tissue injury and repair mechanisms, as well as by studies demonstrating that women with endometriosis may have an impairment of their immune systems and inflammatory responses, allowing for the development of endometriotic lesions (3–6).

The role of macrophages and their relation to endometriosis has been the subject of multiple studies (7, 8). McLaren *et al.* (9) showed that macrophages are highly expressed in the peritoneal fluid of women with endometriosis, stimulating angiogenesis and aiding in the implantation of endometriosis in the peritoneal cavity. Subsequent studies have confirmed macrophages' involvement in creating a positive loop for the establishment of endometriosis via the secretion of macrophage inflammatory protein-1α and -2, IL-6, TNF-α, and vascular endothelial growth factor (10). A combination of markers that included macrophage chemotactic protein-1 and macrophage migration inhibitory factor has been reported as a possible noninvasive test for endometriosis in some women (11).

In very recent years, a nonbiased approach to the study of endometriosis has been undertaken using genomics and proteomics, with the hope that differences between women with and without disease may shed light on novel pathways of disease and possibly lead to the discovery of biomarkers. Families of genes associated with the immune system and inflammatory pathways, cell adhesion, and extracellular matrix remodeling have been among those reported to be differentially expressed in endometriosis (12, 13). Proteomic analyses of peritoneal fluid have confirmed that inflammatory proteins, such as S100-A8, α1-antitrypsin, and apolipoprotein A-1, are elevated in women with endometriosis, but none of these proteins can be used as a biomarker for clinical diagnosis at this time (14, 15). Others have reported dysregulated gene and protein expression in eutopic and ectopic endometrium in women with endometriosis (16, 17), but again these were not a basis for a minimally invasive test of endometriosis (18).

Few studies have applied proteomic techniques to the analysis of plasma from women with endometriosis (19–21). Using high-throughput techniques, these studies have reported promising preliminary findings of differential protein expression in diseased women, but all have acknowledged the severe limitation of not being able to readily identify the proteins discovered.

The aim of the present study was to apply novel, state of the art methods of protein expression analysis using two-dimensional difference gel electrophoresis (2D-DIGE) to analyze serum from women with endometriosis. By allowing for multiple comparisons across gels, 2D-DIGE minimized gel to gel variation, increasing the accuracy and reproducibility of results. Using sophisticated spot analysis software that assigns statistical confidence to spot differences observed, we were able to hone in on protein spots of greatest interest for subsequent identification by mass spectrometry. Our goal was to identify differences in protein expression that may provide a deeper insight into the pathophysiology of endometriosis and to potentially identify novel biomarkers of endometriosis in serum.

Materials and Methods

Samples

After obtaining written informed consent, serum was collected from reproductive age women with pain and/or infertility who were undergoing laparoscopic evaluation. Venous blood was collected preoperatively, the samples were centrifuged for 10 min at 3.500 $\times$ *g*, and the supernatant was aliquoted and stored at -70 C until analysis. Samples were collected at the University of Pennsylvania (Philadelphia, PA) in the years 2003–2005 after study approval by the Institutional Review Board. For surgical staging of endometriosis, if present, the Revised Classification of the American Society of Reproductive Medicine (22) was used. As a control group, we used serum from women who underwent laparoscopy for tubal ligation (n = 23), tubal re-anastamosis (n = 2), or elective bilateral oophorectomy (n = 1) who were asymptomatic and surgically confirmed to be free of endometriosis. There were no statistical differences in menstrual cycle phase of serum collection, use of oral contraceptives or progesterone, or GnRH analogs between groups.

Pools

We randomly selected samples from each disease stage and pooled equal aliquots together. Four serum pools were thus investigated regarding differences in protein abundance: pools I/II (minimal/mild, n = 20), III (moderate, n = 16), and IV (severe, n = 20) and the control pool (n = 20).

Depletion of serum pools

We used a commercially available multiple affinity spin column, MARS Hu-14 (Agilent Technologies, Santa Clara, CA) for depletion of the 14 most highly abundant proteins from the serum pools. In the Supplemental Data (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org), we describe the procedure of protein depletion according to the user manual with additional modifications as well as the

subsequent sample concentration and protein quantification procedures.

Protein labeling

To facilitate the inter-pool and inter-gel comparisons, we used the CyDye fluorescence dyes Cy2, Cy3, and Cy5 (GE Healthcare, Piscataway, NJ). Each dye has different absorption and emission spectra allowing for differentiation between multiple samples on the same 2D gel. The labeling procedure was undertaken according to the instruction manual from GE Healthcare for Amersham CyDye DIGE Fluors (minimal dyes), and 25 μg of each depleted sample was diluted in lysis buffer and labeled with 170 pmol CyDye fluorescence dye. The depleted samples were run in multiple permutations on multiple gels. Each gel was comprised of one internal standard (made by mixing equal amounts of protein from each pool) always labeled with Cy2 and two different pools each with Cy3 and Cy5. Each of the four pools was separated four times, twice labeled with Cy3 and twice with Cy5. This cross-labeling equalizes differences in fluorescence intensity among the three fluors, and the use of an internal standard allows for the accurate detection of small differences in protein abundance between pools, while minimizing inter-gel variations.

Isoelectric focusing

For the separation of proteins in the first dimension, 18-cm immobilized pH gradient (IPG) strips (GE Healthcare) with a nonlinear range of pH 3–11 were used. Isoelectric focusing and equilibration of the IPG strips before SDS-PAGE were undertaken as previously described (23).

SDS-PAGE

For separation in the second dimension, we used 10–20% Tris-tricine gradient gels as previously described by Schägger (24). For SDS-PAGE, we used an Ettan DALTtwelve separation unit (GE Healthcare). The electrophoresis was started with 1 W per gel for 1 h and then increased to a power of 8–12 W per gel.

Imaging and analysis

The gel imaging was carried out as previously described (23).

Statistical analysis

To minimize false-positive results, we predetermined the following criteria: 1) intergroup differences between protein spot abundance had to exceed a factor of ± 2.0 and 2) the P value of the corresponding equalvariance two-tailed Student's t test had to be lower than 0.05. To pare down the number of potentially interesting spots, we focused on those that either increased with severity of disease or decreased with disease severity, because these were more inclined to be clinically meaningful. All spots that fit these criteria were marked as potentially interesting and were picked as gels for further identification.

Mass spectrometric analysis

Spot picking, protein excision, and in-gel digestion were carried out as previously described (25) using trypsin from porcine pancreas (Sigma-Aldrich, Vienna, Austria) for the standard identification and chymotrypsin from bovine pancreas (Sigma-Aldrich) for identification of vitamin D-binding protein (DBP) allele products.

Protein digests were analyzed using MALDI TOF/TOF 4800 Plus analyzer (Applied Biosystems, Foster City, CA) for standard identification and nano-scale liquid chromatography-electrospray ionization-mass spectrometry (MS) (LTQ Orbitrap XL; ThermoScientific, San Jose, CA) equipped with a nanospray interface and coupled to an UltiMate 3000 system (Dionex, Sunnyvale, CA) for identification of DBP allele products. Tandem MS (MS/MS) spectra were searched against a human database using Mascot (Matrix Science, London, UK) and Sequest (ThermoScientific) algorithm. For Mascot search (standard identification), we used the NCBInr database for *Homo sapiens*. Protein scores higher than 63 indicate identity or extensive homology ($P < 0.05$). For the Sequest search, the identified peptides were further evaluated using charge state *vs.* cross-correlation number (Xcorr) as previously described (25). The criteria for positive identification of peptides were Xcorr higher than 1.5 for singly charged ions, Xcorr higher than 2.0 for doubly charged ions, and Xcorr higher than 2.5 for triply charged ions. Fixed modification was carbamidomethylation of Cys residues, and variable modifications were Met oxidation and neutral loss on Ser and Thr residues of NeuNAc-Hex-HexNAc (656.6 Da) and Hex-HexNAc (365.4 Da).

Validation of DBP allele products in single specimens

The validation of DBP allele products in single samples was accomplished by separation on gels as described above, with the following modifications. Single samples were depleted using ProteoPrep Blue albumin and IgG depletion kit (Sigma-Aldrich), and 81 single samples were analyzed: 20 with disease stage I (minimal), 20 with stage II (mild), 17 with stage IV (severe), and 24 control samples. For isoelectric focusing, 18-cm IPG strips with a nonlinear range of pH 3–5.6 were used. Before isoelectric focusing, 25 μg of each depleted sample was labeled with one of the CyDye dyes and investigated once on 2D-PAGE.

DBP forms a chain of three spots when separating in 2D gels due to differences in amino acid sequence and glycosylation of the allelic forms. The allele products found in single samples could be classified according to the pattern of the respective spots on the 2D gel. For confirmation, the volumes of the respective spots containing the DBP allele products were calculated using DeCyder differential analysis software. The relative ratio of the spots containing GC*2 and GC*1F/GC*1S was calculated for relative quantification of the DBP allele products present in the sample. The differences in expression of allele products of single specimens were compared between groups using χ^2 (GraphPad Prism version 4).

Results

To investigate differences in abundance of serum proteins in women with and without endometriosis, we performed 2D-DIGE comparisons between women of different disease stages. After serum depletion, the proteins of the stage-specific pools were labeled with CyDye dyes, separated using 2D-PAGE, in-gel digestion with trypsin and identified using mass spectrometry. Each pooled sample was analyzed four times by running it on four separate

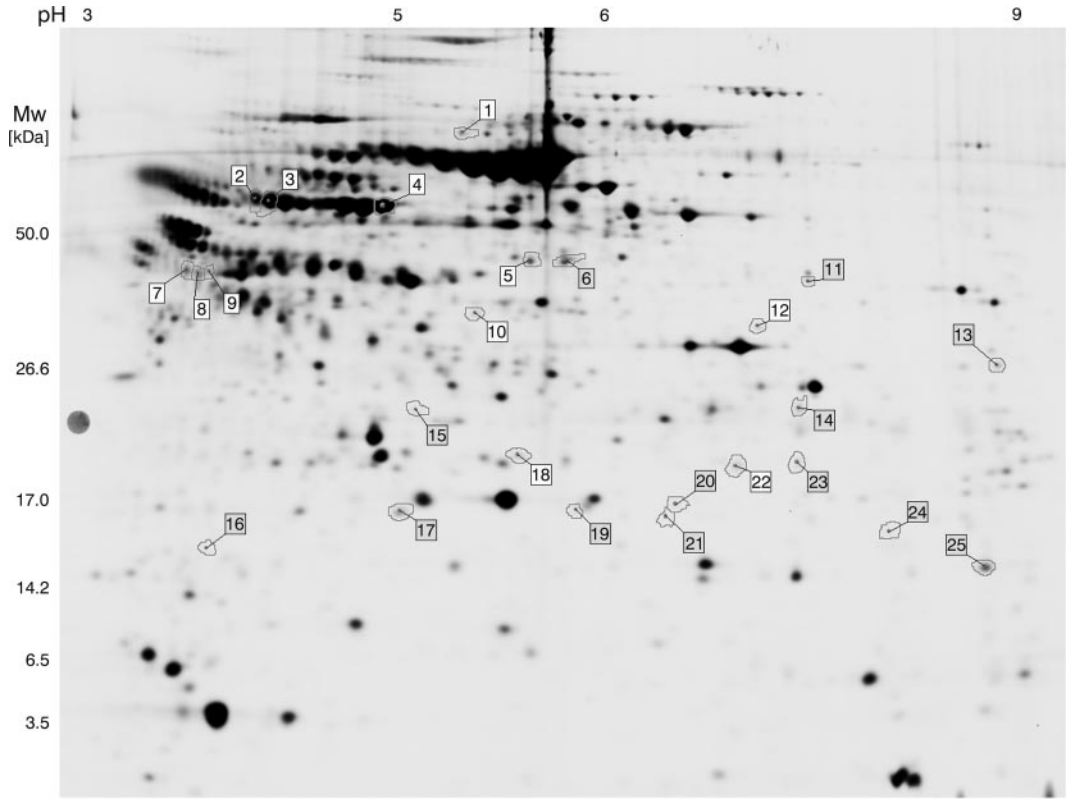

FIG. 1. 2D gel image of serum depleted with Agilent's Hu-14. The *marked spots* differ statistically significantly by a factor of at least 2 between one of the endometriosis pools and the control pool. *White marked spots* were highest in the severe (stage IV) and moderate disease stage (stage III). *Gray marked spots* were highest in the control pool.

gels. We found 25 protein spots with a significant difference in abundance between diseased and disease-free subjects (Fig. 1); 12 spots showed an increase in abundance with severity of endometriosis, 13 spots a decrease in abundance with severity. Thirteen different proteins could be identified in the 25 spots using MALDI TOF/TOF mass spectrometry. A series of important acute-phase proteins and complement components were differentially abundant in serum of women with endometriosis (Supplemental Table 1).

The abundance of the entire DBP, found in spot 4 on the gel, was higher in all endometriosis pools by a factor of approximately 3 compared with the control pool. We decided that DBP merited further detailed investigation due to its uniform high abundance in all endometriosis stages and its known action as a macrophage activator.

Because it is commonly known that DBP is a genetic polymorphism in humans (26), we were interested in identifying the specific allele product present in spot 4. Therefore, we changed the digestion enzyme from trypsin to chymotrypsin, yielding a peptide from amino acids 411–422 (numbered according to the secreted protein, without the signal sequence) that enables the differentiation of the three major allele products (GC*1F, GC*1S, and GC*2) and their possible glycosylation state (Fig. 2A). Using nano-scale liquid chromatography-electrospray ionization-MS, we were able to detect the 1310.55-Da peptide corresponding to 411-KAKLP*D*ATP*K*EL-422 (Sequest Xcorr score, 2.559) which is the specific amino acid sequence of the DBP allele product GC*2. The MS/MS spectrum is shown in Fig. 2C.

It has been reported that the three major allele products differ in their isoelectric point (pI) due to alterations in their amino acid sequence and in their possible glycosylation state (27). Therefore, we hypothesized and subsequently confirmed that the other two major DBP allele products are contained in spots lateral to spot 4 in the 2D gel (Fig. 2B). Spot 4α contained the DBP allele product GC*1F unambiguously identified by the peptide 411-KAKLPDATPTEL-422 (Fig. 2D) (molecular mass, 1283.72 Da; Xcorr score, 3.565) as well as GC*1S identified by the peptide 411-KAKLPEATPTEL-422 (molecular mass, 1297.74 Da; Xcorr score, 2.839). Underlined letters indicate amino acid exchange between allele products. Spot 4β likewise contained GC*1F and GC*1S, however, with both proteins glycosylated at the threonine at position 420, accounting for the separation of 4α and 4β. During MS/MS fragmentation, we observed neutral losses of 291.13 Da $(NeuNAc)_1$, 453.13 Da $(NeuNAc)_1(Gal_1)$, and 656.33 Da $(NeuNAc)_1(Gal)_1(GalNAc)_1$, which indicated that the GC*1F (Fig. 2E) and GC*1S specific peptides in spot 4β were modified with a linear $(NeuNAc)_1$-$(Gal)_1$-$(GalNAc)_1$ trisaccharide, as previously described (28).

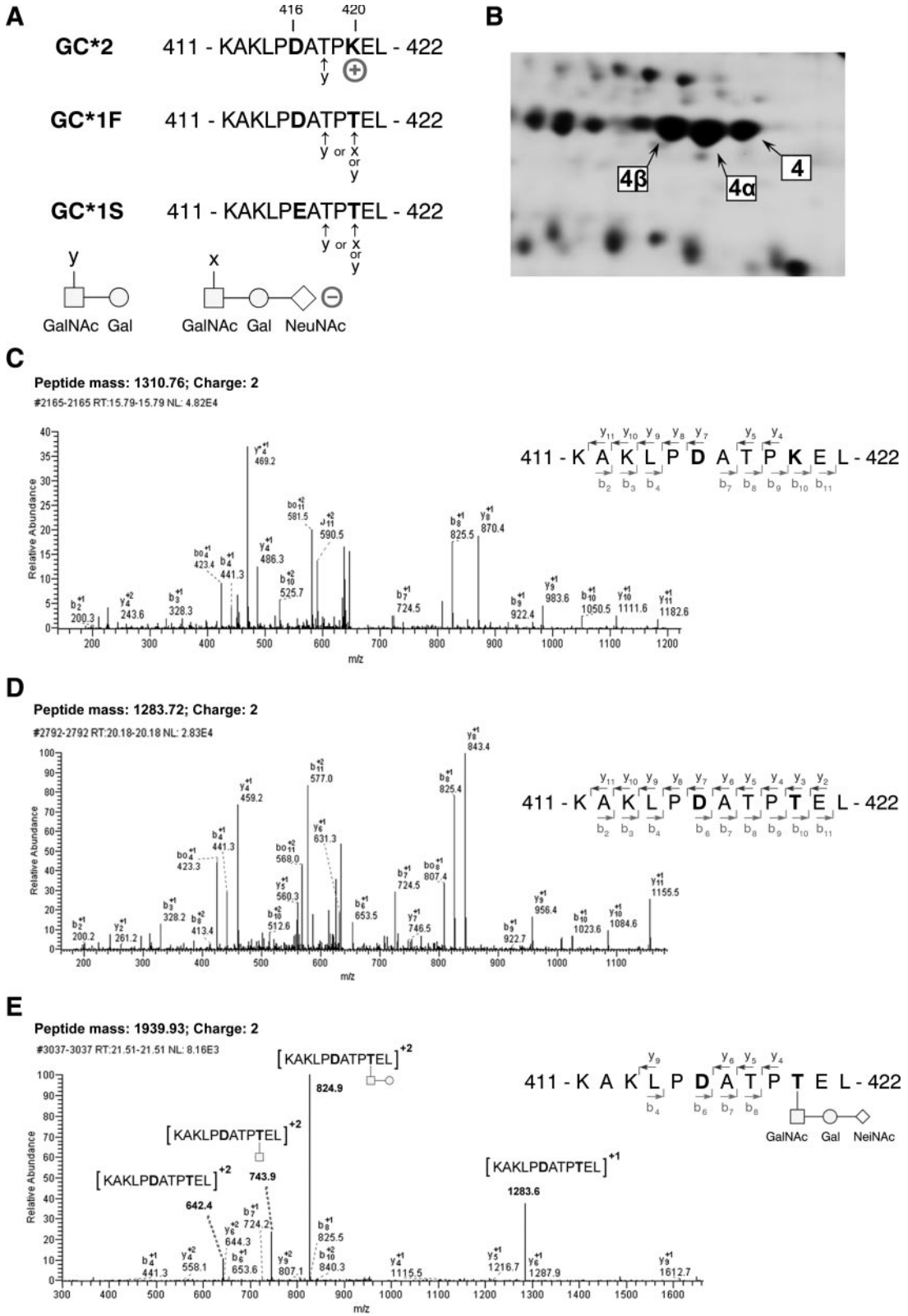

FIG. 2. Mass spectrometry identification of the three major DBP allele products (GC*1F, GC*1S, GC*2). A, Known primary structures of the three major DBP allele products differing at two positions, 416 and 420. *Arrows* denote sites of possible modification with (*x*) tri- or (*y*) disaccharide. B, Detail of Fig. 1. Spot 4 contained the DBP allele product GC*2; spot 4α contained GC*1F and GC*1S unmodified; and spot 4β contained GC*1F and GC*1S modified with the linear trisaccharide. C, MS/MS spectrum of the GC*2-specific amino acid sequence identified in spot 4. D, MS/MS spectrum of the GC*1F-specific amino acid sequence identified in spot 4α. E, MS/MS spectrum of the GC*1F-specific amino acid modified with the linear trisaccharide identified in spot 4β.

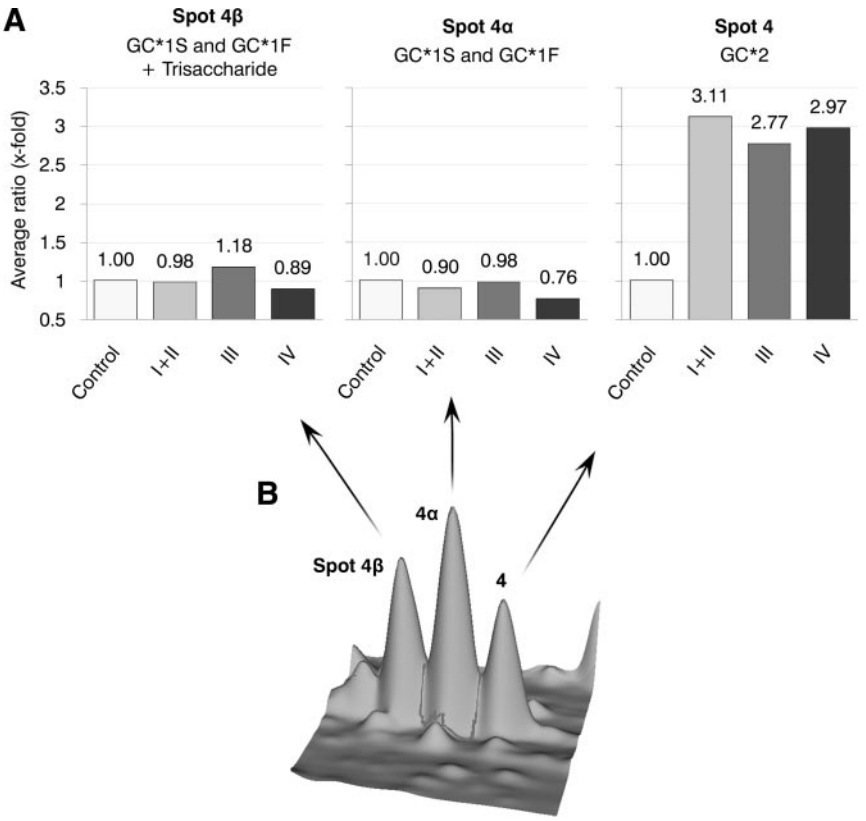

FIG. 3. A, Average ratio of DBP allele products in the endometriosis pools relative to the control pool in the three spots belonging to the DBP chain. Corresponding *P* values for between group differences of spot 4 can be seen in Table 1. B, Three-dimensional rendered detail of the three DBP spots illustrating the spot volume and the quality of separation using a pH range from 3 to 11 for isoelectric focusing.

This observation, that DBP is seen as three separate spots with differing pI values, as described by Arnaud and Constans (29), can be explained by two factors. First, the GC*2 allele product in spot 4 has a higher pI (pI 5.1) than the GC*1 variants in spot 4α (pI 4.94 and 4.95) because of the exchange from threonine to lysine at position 420. Second, the NeuNAc acidifies trisaccharide glycosylated GC*1 allele products resulting in a third distinct spot, 4β (pI 4.84 and 4.85), on the 2D gel. Because of the almost equivalent pI of GC*1F and GC*1S, we were not able to differentiate between those two allele products on the basis of 2D gels.

The electrophoretic separation and subsequent identification of the allele products allowed us to determine that only GC*2 seen in spot 4 showed a difference between endometriosis pools and the control group, as previously discussed (Fig. 3). However, the spots 4α and 4β containing GC*1 allele products did not show significant differences in expression between any pools, nor did the total amount of DBP (combined density of spots 4, 4α, and 4β). This is consistent with the findings of the study by Borkowski *et al.* (30).

Our subsequent aim was to validate the allele products of DBP in single serum samples. The classification of the allele products was performed according to the pattern of spots on the 2D gel. Three combinations were observed: just the GC*1 allele products were present (spots 4α and 4β) (Fig. 4A), or both allele products were present (all three spots) (Fig. 4B), or just the GC*2 allele product was present (spot 4) (Fig. 4C).

In those cases when both allele products were present, we evaluated their relative ratio to each other to verify whether differences between disease stages occur. For calculation, we used the ratio of spot volume 4 to 4α and excluded the volume of spot 4β in the calculation because it is overlaid by a small α1-antitrypsin spot. The mean relative ratio of spot 4 to spot 4α in all single samples expressing both allele products was 1.20 with a SD of $\pm$0.45 (Supplemental Fig. 1). No statistically significant difference between disease stages and the control group was observed. Therefore, we considered all samples that contained both allele products to be a homogeneous group with equal expression of the two alleles.

As depicted in Fig. 5, the distribution of allele products among single specimens was significantly different between the groups evaluated (stage I/II, stage IV, and control) ($P = 0.006$). GC*2 appeared more frequently in the endometriosis samples than in the control group. Of note, no subject in the control group expressed the GC*2 allele product alone, whereas in endometriosis subjects, the expression of GC*2 alone was observed in 17.5%. Conversely, 79.2% of the single specimens in the control pool expressed GC*1 alone compared with 50% of stage I and II and 23.5% of stage IV, respectively.

Discussion

In this study, we found a number of proteins that differed in their abundance in serum between women with and without endometriosis. We confirmed that the methods applied, serum immunodepletion, 2D-PAGE, and mass spectrometry, do indeed permit an insight into the low abundant protein fraction of serum.

Our findings are consistent with those of previous reports showing differentially expressed proteins of the complement system, as well as other acute-phase proteins,

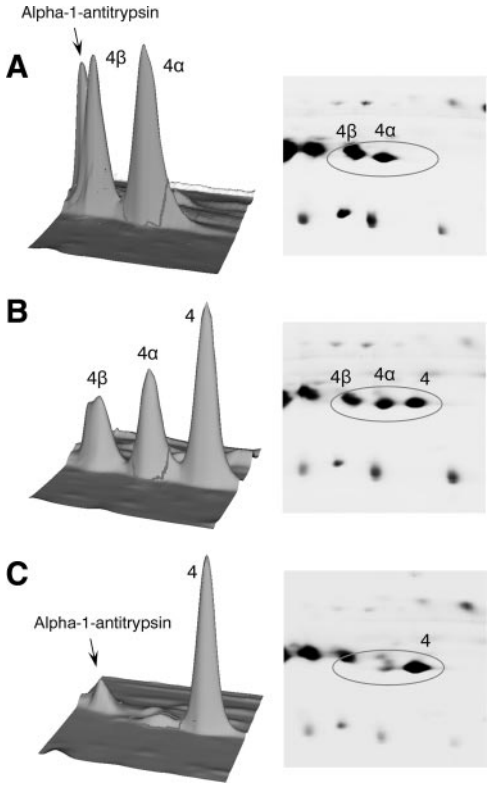

FIG. 4. Appearance of DBP spots in single samples. Three combinations occurred: only GC*1 allele products (spot 4α and 4β) (A); both allele products (all three spots) (B); or only the GC*2 allele product (spot 4) (C).

in women with compared with women without endometriosis (15, 19, 31).

Our most impressive discovery is the differential abundance of a specific allele product of DBP, GC*2. The expression of the GC*2 allele product was 3-fold higher in all endometriosis pools compared with the control pool. Single sample analysis showed that in the control group, no subject expressed only the GC*2 allele product, and approximately 20% of control women expressed both GC*1 and GC*2, implying that the latter were heterozygous carriers of the GC*2 polymorphism. This is consistent with the known carrier rate in the population (32). In contrast, in the endometriosis group, nearly 18% of women expressed only the GC*2 allele product, and 32.5 and 58.8% expressed GC*2 in combination with GC*1 allele products (heterozygous carriers) in stage I/II and stage IV disease, respectively.

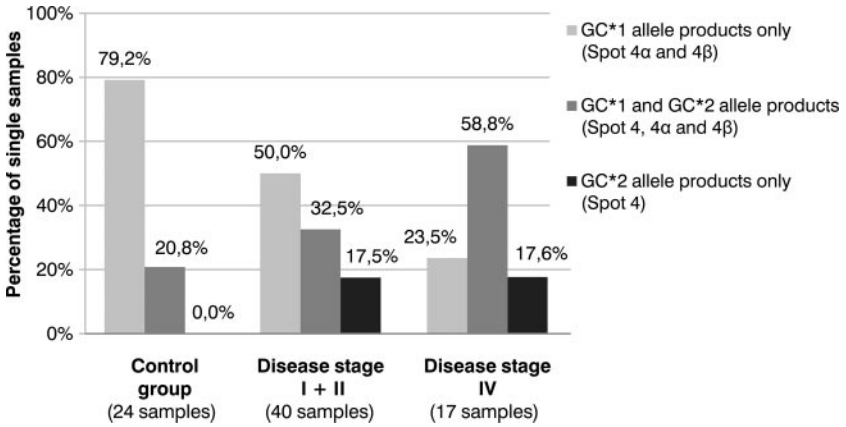

FIG. 5. Frequency of the allelic forms observed in the depleted single samples allocated to the disease stage. The distribution was significantly different between the groups evaluated (stage I/II, stage IV, and control), $P = 0.006$.

To our knowledge, there have been only two studies that have evaluated DBP in endometriosis. Borkowski *et al.* (30) compared total concentrations of DBP in serum and peritoneal fluid of women with and without endometriosis and found no differences. However, this study was unable to differentiate between the different allele products of DBP, as we did.

The other study of DBP used 2D PAGE and reported that one isoform of DBP was significantly lower in the peritoneal fluid, but not in plasma, of women with endometriosis compared with controls (33). The investigators did not identify which of the allele products of DBP was differentially expressed, performing no mass spectrometric analyses and relying only on the relative position of the protein spot on the gel, thus limiting possible comparisons between their results and ours.

Besides acting as a transporter for vitamin D metabolites, DBP is the most potent activator for macrophages in the form of Gc protein-derived macrophage-activating factor (GcMAF) (27, 34). The three major phenotypes of DBP (GC*1S, GC*1F, and GC*2) differ in their primary structure at two positions, 416 and 420, and, consequently, in their glycosylation state. The macrophage-activating function depends on the glycosylation state, *i.e.* most likely determined by a single GalNAc resulting when DBP is deglycosylated by T- and B-cell glycosidases (34). The GC*1 allele products are glycosylated at a total rate of 10–30%, whereas the GC*2 allele product is glycosylated at a rate of 1–5% (28). Thus, the form of DBP encoded by the GC*1 allele is much more readily converted to GcMAF, whereas that encoded by GC*2 is converted hardly at all.

As described above, we found that a much higher percentage of women with endometriosis expressed the GC*2 allele product, both as the only protein expressed (in approximately 17.5%, independent of stage) or in combination with the GC*1 allele products (in upwards of 59%), whereas none of the women in the control group expressed only GC*2 allele products. It follows, then, that those with only GC*2 allele products, disproportionally represented in the endome-

triosis group, have a much reduced capability to convert DBP to GcMAF, the critical macrophage activator. We speculate on the clinical implications of this difference in more detail below.

Macrophages are a key component of the immune system as a first defense against foreign pathogens. Upon activation, macrophages develop the capacity for chemotaxis and phagocytosis and are also mediators of the inflammatory response due to their secretion of growth factors, cytokines, and angiogenic factors (35). Although the absolute number of peritoneal macrophages appears to be increased in women with endometriosis compared with controls, previous studies have already speculated that these macrophages may not be competent scavengers of the refluxed endometrial cells in endometriosis (36, 37).

It has been shown that the GcMAF can activate the scavenger function of macrophages and superoxide production (34, 38) without inducing an advanced cytokine release from macrophages (39). In other words, the activation of the phagocytotic action of macrophages via this pathway appears to be separate from the macrophage-induced inflammatory response. This was further supported by Ma *et al.* (40) who demonstrated that the phagocytosis of particles by macrophages was not a prerequisite for macrophage-mediated proinflammatory cytokine production and, conversely, that secretory products such as TNF-α and ILs were not released from macrophages as a result of particle phagocytosis. Apoptotic cells engulfed by macrophages inhibit inflammatory responses from the phagocytic cells (macrophages) that ingested them. In contrast, cells that die pathologically (necrotic cells) do not down-regulate the macrophage inflammatory response (41). This evidence for two, noncompeting functions of macrophages is consistent with our theory that the inability to adequately activate macrophages via DBP due to overexpression of the GC*2 allele in women with endometriosis affects the scavenger function of macrophages and may allow for the survival and implantation of ectopic endometrial tissues in the peritoneal cavity, although not affecting the macrophage-mediated inflammatory response.

The inability to clear endometrial cells derived from the eutopic endometrium and arriving into the peritoneal cavity via menstrual reflux due to some deficiency in the immune system has long been purported. Based on the findings of our study, we speculate that the immune defect may lie, at least in part, in the inability to sufficiently activate macrophages' phagocytotic function in those carrying the GC*2 allele of DBP. Although we acknowledge the relatively small study population, based on our promising findings, we feel that future studies evaluating specific DBP polymorphisms as a risk factor for endometriosis in larger populations of reproductive age women are warranted. In addition, further studies are needed to determine whether the activation of macrophages via targeted immunotherapy in affected women might form the basis of a novel treatment strategy for endometriosis.

Acknowledgments

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This project was supported by the Science Foundation of the Austrian National Bank Grant 12724, Vienna, Austria.

Disclosure summary: The authors have nothing to disclose.

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