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Serum and peritoneal evaluation of vitamin D-binding protein in women with endometriosis*

Stężenie białka wiążącego witaminę D w surowicy i płynie otrzewnowym kobiet z endometriozą

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Jacek Borkowski[1A], Grzegorz B. Gmyrek[2BCDEFG], Jan P. Madej[3A], Wojciech Nowacki[3A], Marian Goluda[4D], Marian Stanisław Gabryś[4D], Tadeusz Stefaniak[3D], Anna Chełmońska-Soyta[2,3D]

[1] University School of Physical Education, Department of Biochemistry, Wrocław, Poland
[2] Laboratory of Reproductive Immunology, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland
[3] Wrocław University of Environmental and Life Sciences, Faculty of Veterinary Medicine, Wrocław, Poland
[4] Second Department of Obstetrics and Gynecology, Wrocław Medical University, Wrocław, Poland

Summary

Introduction: Vitamin D-binding protein (also called DBP or Gc-globulin) is recognized as a multifunctional protein involved in the actin scavenger system, the transport of vitamin D sterols, and the modulation of immune and inflammatory responses. This study evaluated total serum and peritoneal concentrations of vitamin D-binding protein in women with endometriosis, known as an inflammation-associated disease.

Materials/Methods: The total concentration of DBP was measured with an enzyme-linked immunosorbent assay (ELISA) using a polyclonal antibody raised in a goat immunized with human DBP. Serum and peritoneal fluid were collected from women with endometriosis (n=26) and from patients with benign gynecological conditions serving as a control group (n=17).

Results: In general, the vitamin D-binding protein concentration was higher in serum than in peritoneal fluid. Women with endometriosis had higher serum but lower peritoneal levels of DBP compared with the control group; however, no significance was noted. When the endometriosis group was divided with regard to severity, an insignificantly higher serum level of DBP was observed in advanced endometriosis compared with the mild form of the disease, whereas the peritoneal concentration was not dependent on disease severity.

Conclusions: It is concluded that serum and peritoneal DBP concentrations are not affected in women with endometriosis; however, based on the latest published data, it is possible that both the serum and peritoneal concentrations of vitamin D-binding protein may be dependent on Gc genotype, which results in differential modulation of monocyte/macrophage activity.

Key words: **DBP • ELISA • endometriosis • Gc globulin • goat • immunization • vitamin D-binding protein**

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Streszczenie

Wstęp: Białko wiążące witaminę D (określane także jako DBP lub globulina Gc) znane jest jako wielofunkcyjne białko biorące udział w transporcie pochodnych witaminy D, usuwaniu aktyny czy regulacji odpowiedzi immunologicznej. Przedmiotem badania było określenie całkowitej koncentracji białka wiążącego witaminę D w surowicy i płynie otrzewnowym kobiet z endometriozą, w przebiegu której obserwuje się zmiany w funkcjonowaniu odpowiedzi immunologicznej.

Materiały/Metody: Całkowitą koncentrację DBP mierzono z zastosowaniem testu immunoenzymatycznego ELISA za pomocą poliklonalnego przeciwciała pozyskanego z surowicy kozy immunizowanej ludzkim białkiem DBP. Surowica i płyn otrzewnowy były pobierane od kobiet ze zdiagnozowaną endometriozą (n=26). Grupę kontrolną stanowiły pacjentki z łagodnymi zmianami w okolicy miednicy mniejszej, u których wykluczono obecność endometriozy (n=17).

Wyniki: Niezależnie od badanej grupy pacjentek, całkowita koncentracja DBP była wyższa w surowicy niż w płynie otrzewnowym. U kobiet z endometriozą obserwowano wyższe stężenia DBP w surowicy i niższe w płynie otrzewnowym w porównaniu z grupą kontrolną, przy czym obserwowane wielkości nie były znamienne statystycznie. Po uwzględnieniu stopnia zaawansowania endometriozy, statystycznie nieistotne wyższe stężenia DBP w surowicy zanotowano u kobiet z endometriozą zaawansowaną w porównaniu do kobiet z endometriozą łagodną, podczas gdy koncentracja DBP w płynie otrzewnowym była niezależna od stopnia zaawansowania choroby.

Dyskusja: Wydaje się, że koncentracja DBP w surowicy i płynie otrzewnowym jest niezmieniona u kobiet z endometriozą. Uwzględniając ostatnie dane literaturowe nie jest wykluczone, że koncentracja DBP w surowicy i płynie otrzewnowym kobiet z endometriozą może być zależna od genotypu Gc, co mogłoby się przełożyć na aktywność monocytów/makrofagów.

Słowa kluczowe: **białko wiążące witaminę D • DBP • ELISA • endometrioza • globulina Gc immunizacja**

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Author's address: Grzegorz Gmyrek, Institute of Immunology and Experimental Therapy, Laboratory of Reproductive Immunology, R. Weigla 12, 53-114 Wrocław,Poland; e-mail: gregoire@hot.pl

1. Introduction

Vitamin D-binding protein, initially described as a serum group-specific component (Gc globulin), is recognized as a major plasma protein carrier for vitamin D and its metabolites. In addition, DBP transports fatty acids and is an important component of the actin scavenger system. Gc globulin belongs to the albumin superfamily of binding proteins and show three major phenotype variants: Gc1s, Gc1f, and Gc2 [14,25,32,35]. DBP is synthesized mainly by hepatocytes; however, its differential mRNA expression has been observed in other tissues, including kidney, testis, placenta, lung, heart, spleen, brain, and uterus [8]. Similarly, the highest concentration of DBP is found in plasma, although its expression has been detected in other fluids, such as cerebrospinal fluid, seminal fluid, saliva, and breast milk [14]. The hydrostatic pressure gradient between plasma and the peritoneal cavity may explain its presence in peritoneal fluid [12]. Decreased plasma concentration of vitamin D-binding protein is believed to be a valuable marker in identifying patients with increased risk of mortality after injury due to severe cell or tissue loss. This has been particularly observed during organ dysfunction, massive hepatocyte necrosis, respiratory or hematological failure, and sepsis [14]. In women, serum DBP expression is dependent on estrogen level. Increased serum concentration is observed during pregnancy and hormonal treatment [32]. More recently it has been shown that DBP may modulate inflammatory and immunoregulatory activities, including leukocyte C5a-mediated chemotaxis and macrophage activation. Activated T and B cells release enzymes that transform DBP into macrophage-activating factor (DBP-MAF). This molecule may act as a switch by stimulating macrophage activity at sites of inflammation or inducing cell death in activated macrophages [14,16]. Gc globulin is also considered to be a positive acute-phase reactant, which could suggest that an increased level of DBP may be maintained during an acute-phase response [9].

Endometriosis, an estrogen-dependent disease characterized by an overgrowth of endometrial cells outside the uterus, is believed to be associated with a progressive inflammatory state [4]. The modulation of inflammation is caused by activated macrophages, these being the most numerous cell population in the peritoneal cavity. This results in increased levels of cytokines, growth factors, and other inflammatory mediators

[13] which could ultimately prompt an acute-phase response at the systemic level. Indeed, more recently, increased levels of serum IL-6 and CRP and SAA were noted in women with endometriosis [2,26]. IL-6 may stimulate the expression of haptoglobin, an acute-phase protein, by endometriotic and peritoneal cells [27]. Therefore it could be quite possible that IL-6 prompts the expression of Gc globulin in women with endometriosis at both the local and systemic levels.

Based on the biological properties of Gc globulin and the possible theory recognizing DBP as a positive acute-phase reactant, we hypothesized that aberrant expression in both the serum and peritoneal fluid may take place in women with endometriosis. To the best of our knowledge, an investigation of serum and peritoneal DBP concentrations employing ELISA has not yet been performed. For this reason we used an original, non-commercial ELISA technique employing a polyclonal antibody raised in a goat immunized with human Gc globulin.

2. Materials and Methods

2.1. Reagents and basic equipment

Human Gc globulin (mixed type) for goat immunization was purchased from MP Biomedicals Inc. (Solon, OH, USA); human Gc globulin used to construct an affinity chromatography column and standard curve for ELISA was purchased from Athens Research & Technology Inc. (Athens, GA, USA); complete and incomplete Freund's adjuvants, cyanogen bromide-activated (CNBr) Sepharose 4B, ExtrAvidin-HRP conjugate, Tween 20, and casein (from bovine milk, powder) were purchased from Sigma-Aldrich (St. Louis, MO, USA); DEAE Cellulose, type DE52, was purchased from Whatman (Brantford, UK); phosphate buffer, NaCl, H_2SO_4, H_2O_2, and O-phenylenediamine were purchased from POCH (Gliwice, Poland); glycine-HCl was purchased from A.G. Scientific (San Diego, CA, USA); MaxiSorp 96-well plates were purchased from Nunc GmBH (Wiesbaden, Germany); NHS-LC biotin was purchased from Fisher Scientific Co. (Pittsburgh, PA, USA); and sterile glass tubes containing clotting activator or heparin for serum and peritoneal fluid collection, respectively, were purchased from BD Vacutainer Systems (Plymouth, UK).

2.2. Characteristic of patients

Pre-menopausal women with regular menses (25–32 days in length) undergoing surgical visualization because of pain and/or infertility were enrolled in the study. Twenty-six women, 21 to 50 years old, showed the presence of endometriosis during laparoscopy, later confirmed by histopathological examination. The severity of disease was determined according to the revised American Society of Reproductive Medicine (rASRM) classification [3]. Among the endometriosis patients were women with mild (rASRM stages I–II, n=11) and advanced (rASRM stages III–IV, n=15) stages of disease. The control group consisted of 17 women with benign conditions without evidence of endometriosis. All biological fluids were collected during the proliferative phase of the menstrual cycle confirmed by serum progesterone measurement evaluated by a solid-phase two-site chemiluminescent immunometric assay with IMMULITE 1000 (Diagnostics Product Corporation, Los Angeles, CA, USA). The detection limit of the assay for progesterone was 0.1 ng/ml. The study protocol was approved by the Local Ethics Committee of Wroclaw Medical University and informed consent was obtained from each woman prior to sample collection.

2.3. Sample collection

Venous blood (3–5 ml) from women with and without endometriosis was collected into sterile tubes with clotting activator providing effective separation of blood clot and serum. After clotting, the sample was centrifuged for five minutes at 1000 × g and the obtained serum was transferred to a polypropylene tube (Eppendorf type) and frozen at –20°C until the start of the ELISA. Venous blood was collected between 8:00 a.m. and 10:00 a.m. before the induction of anesthesia and all patients were fasting. Peritoneal fluid (3–5 ml) was collected at the time of surgery into sterile tubes containing heparin as an anticoagulant. After collection, the sample was immediately centrifuged for five minutes at 1000 × g and the supernatant was transferred to a polypropylene tube (Eppendorf type) and frozen at –20°C until the start of the ELISA.

2.4. Preparation of polyclonal antibody against human Gc

Antiserum against human Gc globulin was raised in a goat (a three-year-old male weighing 35 kg). At monthly intervals the goat was injected subcutaneously with 100 μg of human Gc globulin dissolved in 0.5 ml of 0.9% NaCl (pH 7.4) with an equal volume of complete Freund's adjuvant (first five immunizations). Eleven months later, after five prior "booster" injections, during the next four immunizations at monthly intervals, 100 μg of Gc protein dissolved in 0.5 ml of 0.9% NaCl was administered. After that, blood was collected from neck veins into a tube with clotting activator. After clotting, the sample was centrifuged at 800 × g for 10 minutes at 4°C and the serum obtained was collected and stored at –20°C. The protocol of immunization was approved by the Regional Ethics Committee at the Wroclaw University of Environmental and Life Sciences. The immunoglobulin fraction was obtained from the serum by precipitation with ammonium sulfate (one volume of PBS and one volume of saturated ammonium sulfate, pH 7.0, were added to one volume of serum). Then the fraction was dialyzed to 0.05 M phosphate buffer, pH 7.4, and applied on a DEAE cellulose (DE52-type) column. The unbound fraction of immunoglobulin was precipitated by ammonium sulfate and dialyzed to 0.1 M phosphate buffer, pH 7.4, then purified by affinity chromatography. The affinity column was generated by immobilizing 5 mg of human Gc protein on cyanogen bromide-activated Sepharose 4B. The initially purified protein fraction was loaded onto the column and washed with phosphate buffer, pH 7.4. Bound immunoglobulins were eluted at pH 2.2 with 0.1 M glycine-HCl and then the pH was adjusted to 7.0. The progress of purification was estimated after each step by 4–16% SDS/PAGE and Western blot (data not shown). The purified anti-human Gc immunoglobulins were divided into two parts. The first part (Part A) remained unbiotinylated, whereas the second part (Part B) was biotinylated with NHS-LC-biotin by incubation on ice for one hour at a concentration of the biotin-linker equal to 80 ng/ml dissolved in 0.1 M sodium borate at pH 8.5.

Table 1. Serum and peritoneal DBP concentrations (mean ± *SEM* and max. and min. values) in women with endometriosis and the control group

Groups of patients	Vitamin D-binding protein concentrations (µg/ml)					
	Serum			Peritoneal fluid		
	Mean ± SE	Min	Max	Mean ± SE	Min	Max
Control n=17 (serum); n=7 (peritoneal fluid)	424.5±23.5	298.1	652.1	408±23.1	320.5	508.5
Endometriosis (total group) n=26 (serum); n=21 (peritoneal fluid)	449.4±24.4	129.4	658.1	387±24.7	96.3	566.7
Mild endometriosis (rASRM: I–II) n=11 (serum); n=8 (peritoneal fluid)	420.9±30.1	286.9	601.9	388.4±49.6	96.3	566.7
Advanced endometriosis (rASRM: III–IV) n=15 (serum); n=13 (peritoneal fluid)	470.3±36.0	129.4	658.1	386.5±27.5	203.8	555.5

Both parts of the purified polyclonal antibody against human Gc globulin (unbiotinylated and biotinylated) were stored at –20°C until the start of the ELISA.

2.5. ELISA quantitation of serum and peritoneal Gc protein

ELISA evaluation of Gc protein in the serum and peritoneal fluid of the analyzed patients was performed in 96-well microtiter plates. Polyclonal antibody (Part A) diluted 300-fold (experimentally established in three independent experiments) in 100 µl of 0.05 M carbonate buffer (pH 9.6) was coated onto a plate and incubated for 6 hr on a shaker set at 10 rpm. After washing, 100 µl of the investigated sample (final dilutions in PBS with 0.05% Tween 20 and 0.1% casein of serum and peritoneal fluid were 56×10^3 and 5×10^3 times, respectively) was put onto the plate together with the standard sample (purified human Gc globulin purchased commercially) and incubated at room temperature for one hour. After that the plate was washed four times in PBST and then biotinylated polyclonal anti-human Gc antibody (Part B) diluted 300-fold (experimentally established in three independent experiments) was put onto the plate and incubated at room temperature for one hour. After incubation the plate was washed four times in PBST and ExtrAvidin-HRP conjugate (diluted 1000x) was added to the plate and incubated for one hour at room temperature. Then the plate was washed in PBST and O-phenylenediamine (0.4 mg/ml) and 0.3% H_2O_2 (v/v) dissolved in 0.1 M citrate buffer (pH 5.0) were added. The enzyme reaction was stopped after five minutes by adding 100 µl of 1 M H_2SO_4 and the absorbance was read at 490 nm (as the primary wavelength) using a Bio-Tek 340 EL spectrophotometer together with KC3 software to calculate the obtained data (both from Bio-Tek Instruments; Winooski, VT, USA). Serum and peritoneal concentrations were calculated by interpolation from a six-point logarithmic standard curve. All the investigated samples and the standard were examined in triplicate.

2.6. Statistical analysis

Calculations of the obtained data was performed with MedCalc software (version 9.3.0.0, Mariakerke, Belgium). The evaluated serum and peritoneal concentrations had normal distributions according to the Kolmogorov-Smirnov test and parametric statistical methods were used (independent sample Student-*t* test and/or ANOVA as appropriate). Concentrations of serum and peritoneal DBP are expressed as mean ± standard error of the mean (*SEM*). $P<0.05$ was deemed as significant for all tests.

3. Results

3.1. Evaluation of the method

In all cases there were detectable serum and peritoneal DBP concentrations and the ranges of the values obtained in all the investigated groups of patients were 0–300 µg/ml (13%), 300–600 µg/ml (80%), and >600 µg/ml (7%). The coefficient of variation (*CV*) showing intra-assay variation of the DBP concentration was less than 10% and the inter-assay variation *CV* was less than 15%.

3.2. Serum and peritoneal DBP levels

The concentrations of serum and peritoneal DBP (mean ± SEM) are presented in Table 1. In general, in both the study (women with endometriosis) and the control group, serum DBP expression (µg/ml) was insignificantly higher than in peritoneal fluid (449.4±24.4 and 424.5±23.5 vs. 387.2±24.7 and 408.6±23.1, respectively). Serum DBP levels in the women with endometriosis (total group) was insignificantly higher compared with the control group (449.4±24.4 vs. 424.5±23.5), whereas the peritoneal DBP concentration (µg/ml) in the patients with endometriosis (total group) was insignificantly lower compared with the control group (387.2±24.7 vs. 408.6±23.1). When the endometriosis group was divided according to severity of disease, higher values of serum DBP were observed in advanced endometriosis (rASRM III-IV) compared with both the mild form of this disease (rASRM I-II) and the control group (470.3±36.0 vs. 420.9±30.1 and 424.5±23.5). In turn, the peritoneal concentration of DBP was insignificantly lower in both the advanced and mild endometriosis groups compared with the control group (388.4±49.6 and 386.5±27.5 vs. 408.6±23.1). The P values obtained from the individual group comparisons are presented in Table 2.

Table 2. Calculation of *P* values from DBP concentration based on comparison of investigated group of patients

Investigated fluid	Comparison of investigated group of patients	P values
Serum	Endometriosis (total group) vs. control group	0.4911
	Mild endometriosis (ASRM I–II) vs. control group	0.9236
	Advanced endometriosis (ASRM III–IV) vs. control group	0.2856
	Mild endometriosis vs. advanced endometriosis	0.3277
Peritoneal fluid	Endometriosis (total group) vs. control group	0.6390
	Mild endometriosis (ASRM I–II) vs. control group	0.7296
	Advanced endometriosis (ASRM III–IV) vs. control group	0.5997
	Mild endometriosis vs. advanced endometriosis	0.9718

4. Discussion

Among the commonly used analytical techniques helpful in evaluating the DBP level in any biological fluid, radioimmunoassay, rocket immunoelectrophoresis, single radial immunodiffusion, turbidimetry, and nephelometry are preferred. However, ELISA gives comparable results (sensitivity) and is commonly accepted [32]. Despite the fact that in this study we employed a non-commercial ELISA version using polyclonal antibody against human Gc raised in goat, it produced results (DBP concentrations) comparable to other non-commercial ELISAs [15,19] and to a commercially available counterpart as well (own information) in which a monoclonal antibody was used.

The serum DBP level in every healthy human is constant from birth, with a range of approximately 300–600 μg/ml and a half-life of about 2.5–3 days. DBP levels do not show race-related differences nor are they related to age [14,19,32]. DBP concentration is not significantly dependent on the anticoagulant (EDTA, citrate, or heparin) used during sample collection [19]. Lower levels of DBP were observed in cord serum and in patients with cirrhosis of the liver [6]. According to Schiodt et al. [31], serum DBP concentration may be a predictive marker in patients with acute liver failure. In turn, serum Gc globulin levels were unchanged in patients with cancer before therapy (ovarian cancer, colon cancer, pancreatic cancer, breast cancer, or myelomatosis) [19]. Our study also showed that total serum and peritoneal DBP concentration (contained within the typical range of 300–600 μg/ml) is not affected in women with endometriosis.

The Gc globulin concentration may be regulated by circadian rhythm, estrogen, and cytokines. Rejnmark et al. [30] noted that both 1,25 dihydroxyvitamin D ($1,25(OH)_2D$) and vitamin D-binding protein have the same circadian rhythm pattern of expression. Both $1,25(OH)_2D$ and DBP show nadirs in the morning followed by increased values during the day. In our study we always performed sample collection at the same time; for this reason, variation due to the differential circadian dependence of DBP concentration was excluded. Estrogen increases the concentrations of both DBP and $1,25(OH)_2D$ [34]; however, in women with endometriosis the serum estrogen level is unchanged compared with women without endometriosis [20]. Similarly, the same peritoneal estrogen levels are observed in both patients with and without endometriosis, although they are higher than in peripheral blood [18]. Bouillon et al. [5] observed positive correlation between serum 1,25-dihydroxyvitamin D_3 ($1,25\text{-}(OH)_2D_3$) and DBP concentrations in normal women and in those taking estro-progestogens. It is unknown whether the $1,25(OH)_2D$ level correlates with total DBP concentration; however, recent data show that plasma levels of $1,25(OH)_2D$ may be dependent on Gc phenotype [22]. This could be interesting in light of findings by Hartwell et al. [17], who noted increased serum levels of $1,25(OH)_2D$ in women with endometriosis.

Among the factors which may influence DBP expression are IL-6 and TGFβ. According to Guha et al. [15], IL-6 may increase the expression of Gc mRNA twofold, whereas TGF-beta decreases Gc mRNA expression, in a dose-dependent fashion up to fivefold. In turn, IL-1 and TNF-alpha have no effect on Gc expression. It is interesting that both IL-6 and TGF-beta are elevated in women with endometriosis, both in serum and peritoneal fluid [21,26,28,29]; however, their influence on Gc expression at the mRNA and protein levels (if any) in women with endometriosis is still awaiting exploration. The modulation of DBP expression by IL-6 suggests that Gc globulin may be considered an acute-phase protein. Guha et al. [15] believe that DBP plays a role as a neutral acute-phase reactant. Dahl et al. [9], however, showed that total Gc globulin concentration displays characteristics similar to haptoglobin, which is believed to be a positive acute-phase reactant. However, the existence of the acute-phase response in women with endometriosis remains controversial. Abrao et al. [1] observed that CRP and SAA were higher in women with advanced endometriosis compared with healthy women, mainly during the first three days of the menstrual cycle. Increased peritoneal levels of complement component 3 (C3) were observed in women with endometriosis as well [33]. On the other hand, Boutten et al. [7] were unable to show any significant differences in peritoneal concentrations of alpha 1-acid glycoprotein, alpha 1-antitrypsin, alpha 2-HS glycoprotein, and albumin in patients with minimal and mild endometriosis. Drews et al. [10] observed a significantly decreased reactivity coefficient (Rc) for α1-antichymotrypsin, but an insignificantly higher one in the peritoneal fluid of women with endometriosis. Similarly, the Rc for α1-antitrypsin was lower in serum, whereas no signi-

ficant differences in peritoneal fluid were found. In turn, Dunselman et al. [11] postulated that the influence of endometriosis on fecundity is probably not related with acute-phase protein synthesis. It cannot be excluded that the levels of both serum acute-phase reactants and/or some cytokines may be unaffected in women with endometriosis; however, correlation between these proteins may take place. Xavier et al. [36] did not notice any changes in serum CRP concentration in women with endometriosis; however, they showed positive correlation between CRP and VEGF in the proliferative and late secretory phases of the menstrual cycle. Thus it is quite possible that despite unaffected concentrations of DBP in women with endometriosis, correlation between both Gc globulin and IL-6 expression and/or some acute-phase proteins may exist.

So far there is only one published paper showing peritoneal and serum expression of DBP in women with endometriosis. Ferrero et al. [12] used 2-D gel electrophoresis to study DBP protein expression in women without and with endometriosis (untreated or using an oral contraceptive). They observed that the relative expression of a DBP isoform (DBPE) is significantly decreased in the peritoneal fluid of patients with endometriosis compared with controls. On the other hand, the isoelectric focusing (pI) and molecular weight (Mw) for DBPE remained unchanged in all the investigated groups of patients (control group and study group). Ferrero et al. [12] concluded that the decreased expression of DBPE may be caused by conversion of DBP to MAF. This seems logical, but on the other hand it is not certain whether the precursor activity of peritoneal Gc corresponds to the total concentration of DBP and/or DBPE. Yamamoto et al. [37] showed that in patients with SLE, the precursor activity of plasma Gc is lost or reduced as a result of deglycosylation of plasma Gc protein by α-N-acetylgalactosaminidase; however, at the same time the concentration of Gc remained unchanged in SLE patients compared with controls. In the study by Ferrero, DBPE did not correspond to the protein product of Gc globulin phenotype due to lack of changes in the isoelectric focusing (pI). This isoform was provisionally identified as native DBP. Ferrero et al. [12] did not observe significant differences in the expressions of other DBP isoforms. Recently, Lauridsen et al. [24] postulated that the mean serum Gc concentration is related to the Gc phenotype. In another study they indicated that differences in osteoclast activity may correlate with Gc phenotype [23].

Conclusion

In conclusion, our study showed that the total concentration of serum and peritoneal DBP is not affected in women with endometriosis; however, based on the present data, it is not excluded that the precursor activity of endometriotic plasma and peritoneal Gc protein may be differential and that diverse Gc phenotypes may exist among women with endometriosis, resulting in differential modulation in monocyte/macrophage activity. We also showed that our model of ELISA based on a polyclonal antibody raised in a goat immunized with human Gc is a valuable method for evaluating the total Gc concentration in different biological fluids.

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