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Dysregulation of GdA Expression in Endometrium of Women With Endometriosis: Implication for Endometrial Receptivity

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Abstract

Glycodelin-A (GdA) has been proposed to represent a potential biomarker of endometrial function, but little is known about its expression during the different phases of the menstrual cycle and under pathological conditions. In the light of its potential importance also in embryo implantation, we aimed to evaluate the expression profile of GdA as well as the presence of different glycosylated glycoforms and the immunolocalization in endometrial tissue from women with endometriosis and in women with proven fertility, at different times during the menstrual cycle. Our results showed that GdA is synthesized by endometrial epithelial and stromal cells, both in healthy endometrium and eutopic endometrium from women with endometriosis, with a profile including several glycosylated glycoforms, differentially expressed in each phase of the menstrual cycle. During the secretory phase, a significant increase in GdA protein expression, with a different glycoforms profile, was observed in endometriotic eutopic endometrium. Protein localization in eutopic endometrial tissue resulted significantly different in comparison with endometrium from women with proven fertility. This study indicate that GdA is a complex glycoprotein including up to 6 different glycoforms specifically expressed during the different phase of the menstrual cycle; in pathologic conditions such as endometriosis, the expression profile is altered possibly related to the impaired endometrial receptivity.

Keywords

endometriosis, glycodelin-A, human endometrium, menstrual cycle, 2-D electrophoresis

Introduction

Endometriosis is one of the most common gynecological disease affecting about 10% of women in reproductive age.[1] This pathology affects woman's reproductive system including ovarian function, oocyte quality, embryo development, and implantation.[2,3] Although its etiology remains unclear, it is generally defined as the presence of endometrial-like tissue outside the uterine cavity and is associated with a variety of different symptoms, including chronic pelvic pain and subfertility.[4]

At moment, the only diagnostic tool for endometriosis is represented by invasive laparoscopic examination. It is therefore not surprising that researchers are actively looking for a biomarker for a noninvasive diagnosis of endometriosis.[5-7] A growing interest is raising toward the study of the expression of glycodelin, formerly known as placental protein 14, a glycoprotein with 4 isoforms (glycodelin A, F, S, and C) having the same protein core but differing in their glycosylation pattern of the 2 N-linked oligosaccharide chains.[8] Glycodelin is a widely expressed glycoprotein whose biological functions, ranging from immunosuppressive properties, mainly mediated by the inactivation of natural killer cells, to sperm-zona pellucida binding inhibitory activity, are determined by its oligosaccharide moieties.[9-11] The glycodelin-A (GdA) isoform is known to be produced by the endometrial glandular epithelium in response to progesterone, human chorionic gonadotropin, and relaxin.[12,13] By immunohistochemistry and *in situ* hybridization, Mylonas and colleagues[14] reported that, in women of proven fertility, GdA is secreted differently by the endometrial glandular epithelium during the menstrual cycle. They reported very low levels of expression in the proliferative

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phase (days 1-14), with a significant increase in the early secretory phase (days 15-22) and a decrease in the late secretory phase (days 23-28). Similar results, although with different sample dating, were reported on uterine flushing by radioimmunoassay.[15] An increase in GdA secretion at the window of implantation has also been reported by a global gene profiling approach.[16]

Several studies on plasma and peritoneal fluids of endometriotic women suggested that an increase in GdA levels could represent, in these biological fluids, a potential biomarker of endometriosis.[7,17,18] On the contrary, immunohistochemical studies on endometrial biopsies suggest a decrease in GdA expression in endometriotic samples after day 22 of the menstrual cycle, in comparison with healthy ones.[5,19] Similar results were obtained by gene profiling on endometrial biopsies at day LH + 8 to LH + 10.[5]

A distinctive trait of GdA resides in its glycosylation profile and sialylation degree. It has been reported that the main differences in sugar moieties among the glycodelin isoforms are mostly at the level of the sialic acid residues and that its immunosuppressive activity is higher with an increased degree of sialylation, as also reported in clinical studies.[11,20,21]

The aim of the present study was to assess the expression of GdA isoforms by applying 2-dimensional (2-D) electrophoresis, immunoblotting, and immunofluorescence analysis in order to depict the specific profile of GdA expression during the menstrual cycle. To this goal, protein expression and the presence of its different glycoforms, resulting from oligosaccharide-linked chains, were evaluated in timed endometrial biopsies taken from patients with endometriosis and from control women with proven fertility.

Materials and Methods

Patients and Specimens

The study was approved by the ethics committee of Siena University. Signed informed consent was obtained from all patients who participated in the study, performed between June 2014 and December 2015, at the Siena University Hospital. All methods were carried out in "accordance" with the relevant national guidelines and regulations and with the Declaration of Helsinki. Endometrial specimens were collected by hysteroscopy, from women of proven fertility undergoing laparoscopy for tubal sterilization (n = 22) and from infertile women affected by endometriosis undergoing laparoscopic surgery (n = 20). The age of the women ranged from 22 to 39 years and was similar between the groups of Caucasian women with and without endometriosis (34.0 ± 3.7 years and 33.1 ± 4.1 years, respectively; $P = 0.2$). The stage of the disease was classified according to the American Society for Reproductive Medicine classification[22] and histologically confirmed by pathologists. Current infections, endocrine disorders, or the use of hormonal treatment within the past 3 months were common exclusion criteria. A complete medical history, physical examination, and transvaginal ultrasound evaluation were performed for each patient. Infertile women affected by endometriosis failed to obtain a pregnancy after 24 months of unprotected sexual intercourses; male factor was excluded in all couples.

All endometrial biopsies were then classified according to the phase of the menstrual cycle on the basis of ultrasonographic features and hormonal profile. Endometrial samples from control women were classified as proliferative phase (n = 6), fertilization window (n = 5), implantation window (n = 5), and late secretory phase (n = 6). Endometrial tissues obtained from endometriotic patients were categorized as proliferative phase (n = 5), fertilization window (n = 5), implantation window (n = 5), and late secretory phase (n = 5).

A fragment of tissue was washed twice with phosphate-buffered saline (PBS), submerged in liquid nitrogen, and then stored at −80°C for subsequent protein analysis, whereas another fragment was fixed for histochemical examination (see below).

Two-Dimensional Electrophoresis

After thawing, endometrial biopsies were washed again in PBS, minced, and solubilized twice, using a conventional 2-D lysis buffer, consisting of 8 M urea, 4% wt/vol 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (Sigma-Aldrich, Milan, Italy), 1% wt/vol dithiothreitol (DTT; Bio-Rad, Danville, CA, USA). After centrifugation at 13 000*g* for 10 minutes, supernatant was collected and stored at −20°C until use. Protein content has been evaluated using the Bradford method with bovine serum albumin (BSA; Sigma-Aldrich) as standard.[23]

For 2-D electrophoresis, 80 µg of total protein was dissolved in a conventional 2D lysis buffer to a final volume of 150 µL and loaded onto Immobiline DryStrips with immobilized nonlinear pH gradient, from pH 3 to 11, of 7 cm length (GE Healthcare, Uppsala, Sweden) containing 2% vol/vol ampholytes (pH: 3-11, IpG buffer; GE Healthcare). Isoelectric focusing was performed as described by Görg et al[24] and Bjellqvist et al[25] using Ettan IPGphor (GE Healthcare). Isoelectric focused strips were equilibrated for 15 minutes with 50 mM Tris–HCl, pH 6.8 containing 30% vol/vol glycerol, 6 M urea, 2% wt/vol sodium dodecyl sulfate (SDS; Sigma-Aldrich), and 1% (vol/vol) DTT and for additional 5 minutes in the same solution containing 2.5% wt/vol iodoacetamide instead of DTT and 0.1% wt/vol bromophenol blue and then placed on a 6% to 16% polyacrylamide linear gradient SDS gels (6.5 cm × 8.5 cm × 1.0 mm) and subjected to electrophoresis in the second dimension at 25 mA per gel constant according to Laemmli.[26]

Western Blotting

Proteins were electroblotted from polyacrylamide gels to nitrocellulose (Bio-Rad Laboratories) overnight at 180 mA e 500 V according to Towbin et al.[27] Membranes were blocked by incubation with 3% wt/vol nonfat dry milk in 10 mM Tris–HCl (pH: 7.5), 0.15 M NaCl, and then incubated overnight with an anti-GdA antibody direct against the peptidic portion of the

glycoprotein (R&D System, Minneapolis, Minnesota) diluted 1:200 in the same buffer containing 0.1% wt/vol Tween-20. After washing, membranes were incubated for 1 hour with an horseradish peroxidase (HRP)–conjugated antigoat immunoglobulin G (IgG) antibody. Immunoreactivity was detected using chemiluminescence reagents (HRP Immuno-Star HRP substrate kit; Bio-Rad). The same nitrocellulose sheets were also incubated with an anti-β-actin antibody followed by the appropriate secondary antibody.

The chemiluminescence signals were captured using a Bio-Rad Chemi-Doc system and quantified using a PDQuest 2D analysis software (Bio-Rad).

Removal of N-Linked Oligosaccharide Chains

N-Linked oligosaccharide chains were released from the protein backbone by peptide N-glycosidase F (PNGase F) from *Flavobacterium meningosepticum* (Sigma-Aldrich). Aliquots of 120 μg of endometrial biopsy lysates were dissolved in 20 mM NaH_2PO_4, pH 7.2, containing 50 mM β-mercaptoethanol and 0.1% (wt/vol) SDS. Samples were denatured by heating to 100°C for 5 minutes. After cooling on ice, the solution was incubated with 0.5% (wt/vol) Nonidet P-40 and PNGase F (5 U/mL) for 16 hours at 37°C. As control, an equivalent amount of sample was incubated under the same conditions in the absence of the enzyme. Both treated and control samples were then analyzed by 1-D and 2D- electrophoresis followed by Western blotting with an antiglycodelin antibody as described above.

Immunofluorescence

For immunofluorescence GdA localization, tissue samples were fixed in 10% neutral-buffered formalin (Bio-Optica, Milan, Italy) at room temperature for 24 hours. Formalin-fixed, paraffin-embedded endometrial tissues and ectopic lesions were sectioned at 4-μm thickness and mounted on Superfrost Plus microscope slides (Fisher Scientific, Ottawa, Ontario, Canada). Tissue sections were then deparaffinized with xylene and dehydrated with ethanol. Sections were treated with PT Module buffer 2 EDTA pH 8 (Thermo Scientific, Waltham, MA, USA) to unmask epitopes. Single- and double-fluorescence approaches were applied by using an indirect procedure. Sections from all samples were incubated in a blocker solution containing 5% goat normal serum in PBS-BSA 1%. Specificity of immunostaining was confirmed by both omission of primary antibody and staining of sections with unrelated antibodies. The slides were incubated for 1 hour at room temperature with the goat anti-GdA polyclonal antibody (1:100; R&D, Abingdon, UK), used according to the manufacturer's instructions. Glass slides were then washed in PBS, and the bound antibodies were revealed by incubation with Alexa Fluor 488-labeled rabbit antigoat IgG antibody (1:100; Thermo Fisher Scientific, Waltham, MA, USA). After washing in PBS, the slides were mounted in ProLong antifade with 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies) to counterstain the nuclei and then observed with a Leica DMB 6000 microscope (Leica, Wetzlar, Germany). Images were captured with an CFTR6500 digital camera (Leica). Fluorescence intensity analysis was performed with LSA software (Leica) and with ImageJ (NIH); statistical significance was assessed.

Statistical Analysis

Data were expressed as mean (standard deviation). Statistical analysis was performed with the software GraphPad Prism version 4.0 (GraphPad Software). Standardized skewness and kurtosis values were used to determine the normal distribution of data. Student *t* test or Wilcoxon signed rank test were used when appropriate. Analysis of variance between groups was performed by 1-way analysis of variance test followed by Bonferroni post hoc comparison test. Statistical significance was set at $P < .05$.

Results

In fertile women, endometrial GdA expression is specifically modulated during the different phase of the menstrual cycle. In fact, by 2-D electrophoresis and immunoblotting, we were able to quantify GdA expression level and definitely analyze the presence of its different glycoforms, resulting from oligosaccharide-linked chains.

Glycodelin-A Expression in Healthy Endometrium

During the proliferative phase, GdA is markedly expressed by the human endometrium (Figure 1A), and 4 main glycoforms are present in this phase, with a barely detectable fifth one (Figure 1E). Glycodelin-A significantly diminished to almost undetectable levels at the fertilization window (Figure 1B), with all 5 glycoforms reduced at the same extent (Figure 1F). Afterward, the protein is newly expressed at the highest level during the implantation window (Figure 1C), when a new sixth more acidic glycoform appears (Figure 1G). Finally, a significant decrease in spot intensity is clearly evident during the late secretory phase (Figure 1D), with 2 main and 2 minor glycoforms still detectable (Figure 1H). Therefore, as summarized in Figure 1 (bottom panel), comparative evaluation of total GdA expression levels throughout the menstrual cycle indicates a peak in protein expression at the implantation window, suggesting a deep involvement of GdA in embryo receptivity.

In order to confirm that the pattern of different glycoforms was due to differential glycosylation, we digested the endometrial samples with N-glycosidase F, an enzyme able to remove all oligosaccharide N-linked chains from glycoproteins. After this treatment, by SDS-PolyAcrylamide gel electrophoresis (PAGE), we detected a GdA band corresponding to 20 kD, whereas in untreated sample, the band showed a molecular weight of about 28 kD (Figure 2A). When the same samples were analyzed by 2-D electrophoresis, a single spot corresponding to a lower PI was highlighted in the deglycosylated

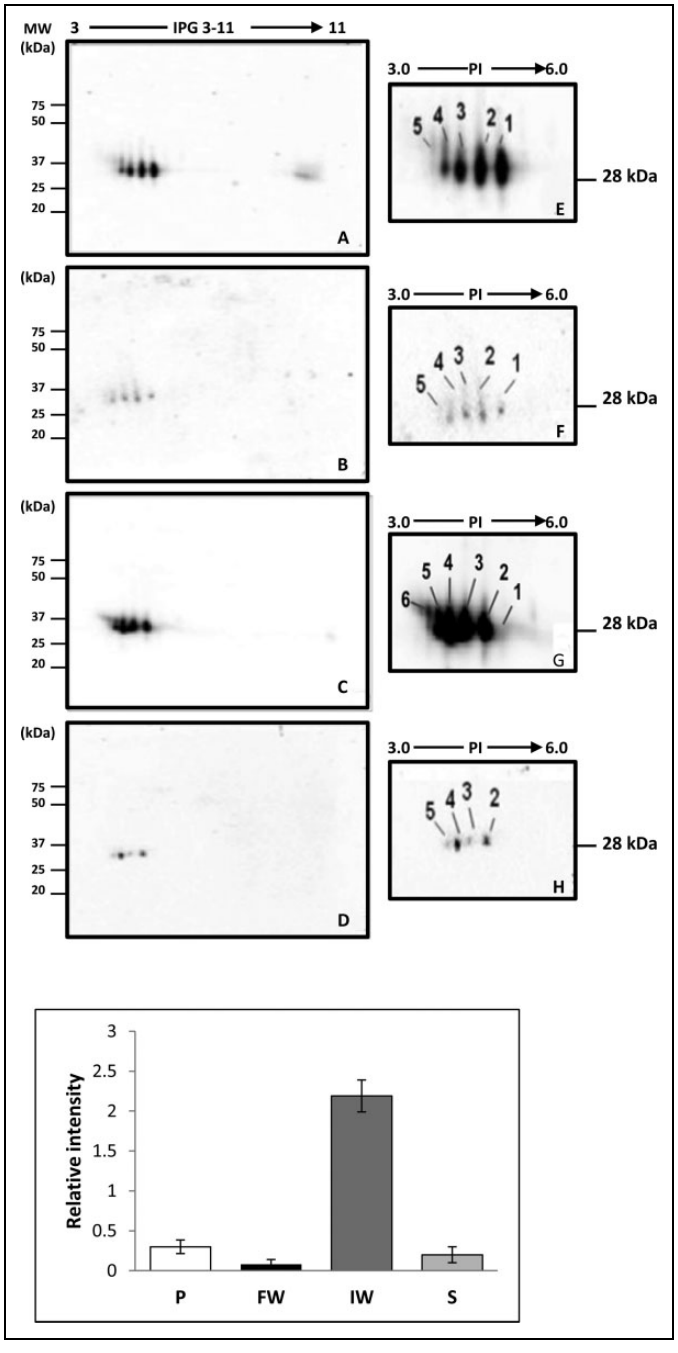

Figure 1. Expression of glycodelin-A (GdA) in the endometrium of women of proven fertility during the menstrual cycle. Upper panel: Immunoblotting detection of GdA after 2-dimensional (2-D) electrophoresis in endometrial tissue demonstrates the presence of several glycoforms. During proliferative phase (A and E), 4 main isoforms are present, with a barely detectable fifth one; at the fertilization window (B and F), all 5 GdA isoforms are significantly reduced, while at the implantation window (C and G), up to 6 glycoforms are expressed at the highest level. In the secretory phase (D and H), an overall significant decrease in spot intensity is evident. Bottom panel: Computer-assisted semiquantitative analysis of the overall relative intensity of the GdA reactive spots. Glycodelin-A intensity was measured (pixel/mm^2) and then normalized with respect to β-actin. The graph shows a significant increase in GdA level at the implantation window, whereas, both at the fertilization and secretory phase, a decrease in GdA expression is evident. Values are expressed as mean (±standard deviation).

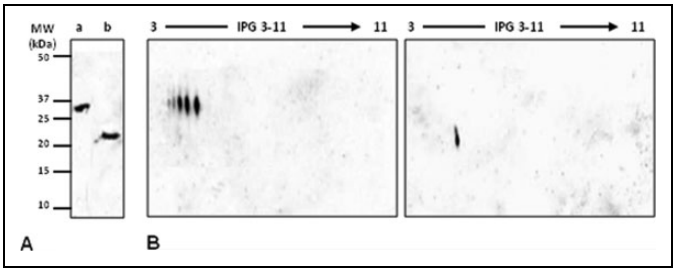

Figure 2. Immunoblot analyses with an antiglycodelin antibody of endometrial biopsies samples with (a) or without (b) N-Glycosidase F treatment, after mono- (A) and 2-dimensional (B) electrophoresis.

sample, confirming that this treatment completely abolished all glycoforms present in untreated protein (Figure 2B). This led us to conclude that the presence of differential isoforms is actually linked to microheterogeneity in the carbohydrate moieties of glycodelin and should be therefore called "glycoforms."

Glycodelin-A Expression in Eutopic Endometrium

A different GdA expression pattern was observed when the same analyses were carried out on eutopic endometrium of women affected by endometriosis. During the proliferative phase, GdA immunostaining was detectable at low level (Figure 3A), with the 5 main glycoforms equally expressed (Figure 3E). Glycodelin-A further diminished at the fertilization window (Figure 3B), when 4 glycoforms are present, while the fifth one was barely detected (Figure 3F). During the implantation window, the protein resulted highly expressed (Figure 3C), with 5 glycoforms clearly visible, whereas the sixth glycoform typical of this phase disappeared (Figure 3G). At the late secretory phase, the high levels of GdA are still observed (Figure 3D), with a strong signals corresponding to 1 to 5 glycoforms, whereas the sixth glycoform, typical of the implantation window, disappeared (Figure 3H). The semiquantitative analysis of the overall protein expression in eutopic endometrium (Figure 4, bottom panel) indicates that the high level of GdA detected at the implantation window remains sustained also at the late secretory phase. Finally, by comparing GdA quantitative expression between healthy and eutopic endometrium (Figure 4), a statistically significant decrease was detected at the proliferative phase in endometriosis ($P = 0.023$), whereas any significant difference was observed during both fertilization and implantation window. On the contrary, at the late secretory phase, a 10-fold increase in GdA expression in the endometrium from patients with endometriosis could be demonstrated ($P = 0.003$). It is possible that this derangement in GdA expression in patients with endometriosis may account for fertility problem affecting these patients.

Glycodelin-A Immunolocalization in Healthy and Eutopic Endometrium

In order to corroborate our data from biochemical analysis, we used immunofluorescence localization of GdA in healthy and

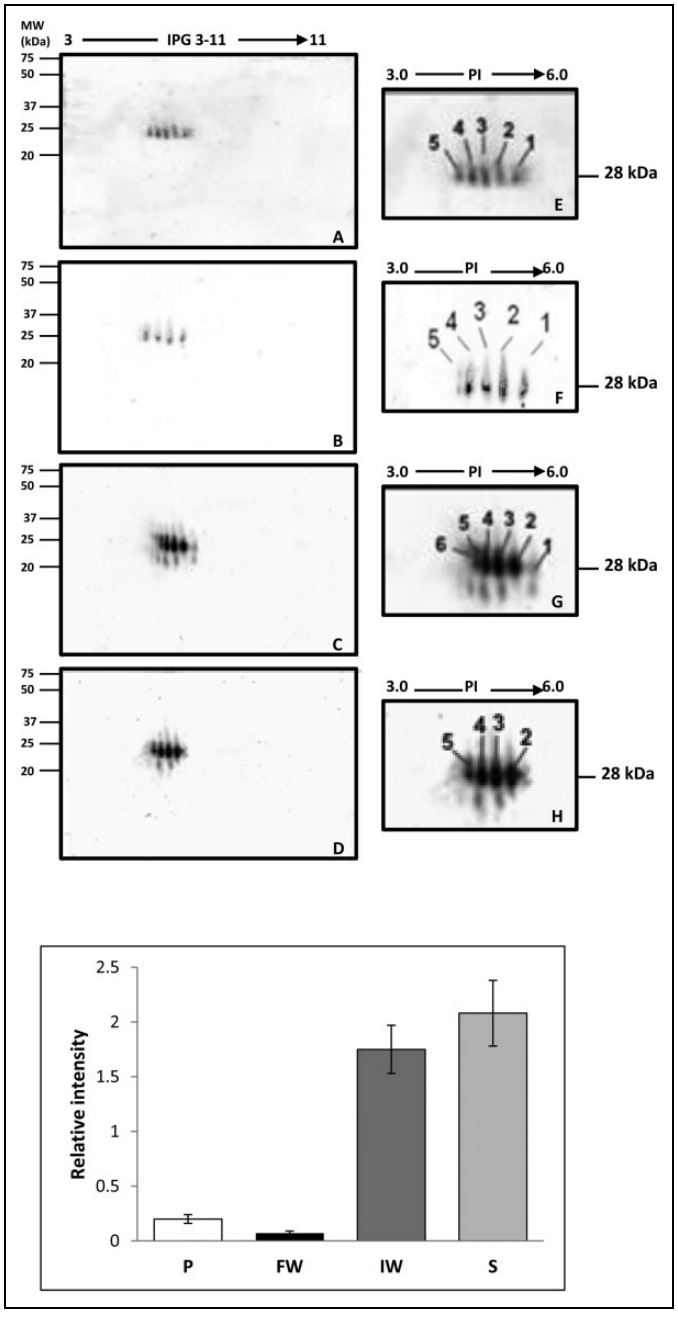

Figure 3. Expression of glycodelin-A (GdA) in endometrium of women with endometriosis during the menstrual cycle. Upper panel: Immunoblotting detection of GdA after 2-dimensional electrophoresis in endometrial tissue demonstrates the pattern of expression of the various glycoforms. During the proliferative phase (A and E), 5 main glycoforms are detectable at low level, while only 4 isoforms are weakly expressed at the fertilization window (B and F). During the implantation window (C and G), only 5 isoforms are highly expressed, with the sixth isoform barely detectable. At the late secretory phase (D and H), a strong signal corresponding to 1 to 5 isoforms is still observed. Bottom panel: Computer-assisted semiquantitative analysis of the overall relative intensity of the GdA in endometrium of women having endometriosis. Glycodelin-A intensity was measured (pixel/mm^2) and then normalized with respect to β-actin. The graph highlights as the significant increase in GdA content at the implantation window remains sustained during the secretory phase. Values are expressed as mean (standard deviation).

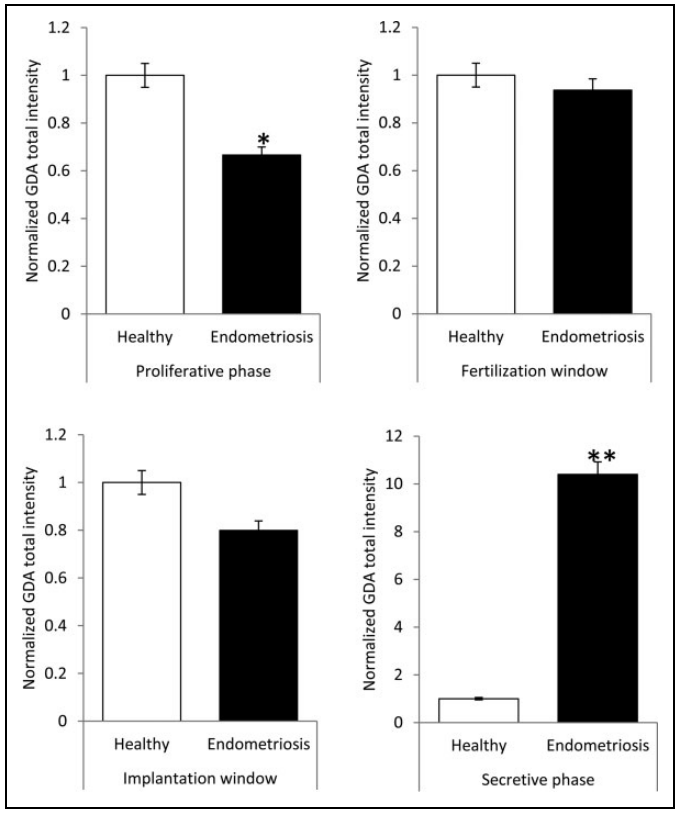

Figure 4. Computer-assisted semiquantitative analysis of the overall relative intensity of the glycodelin-A in endometrium from healthy fertile women and women with endometriosis. By comparing the GdA quantitative expression between healthy and eutopic endometrium, we highlight a significant decrease at the proliferative phase in endometriosis and a 10-fold increase at the late secretory phase. *$P = 0.023$; **$P = 0.003$.

eutopic endometrium throughout the menstrual cycle. In endometrium from women of proven fertility, GdA was present in glandular and luminal epithelial cells as well as in stromal cells during the proliferative phase (Figure 5A). At this phase, a weaker staining was instead highlighted in eutopic endometrium, narrowed to glandular and luminal epithelial cells (Figure 5E). According to data from proteomic analysis, during fertilization window, we observed a weaker staining both in control and in eutopic endometrium, restricted to the glandular epithelium (Figure 5B and F). At the implantation window, an intense staining is present both in healthy and eutopic endometrium, where the stromal cells resulted weakly stained in comparison with the epithelial cells (Figure 5C and G). Finally, in the late secretory phase, GdA staining was mostly restricted to glandular epithelium in the control endometrium (Figure 5D), whereas an intense staining was detected both in glandular and luminal epithelial cells and in stromal cells in the eutopic endometrium (Figure 5H). No staining was observed in the negative control (data not shown). Altogether, immunofluorescence observations and quantification (Figure 5, bottom panel) of GdA expression were in agreement with data on protein expression as revealed by 2-D electrophoresis and immunoblotting.

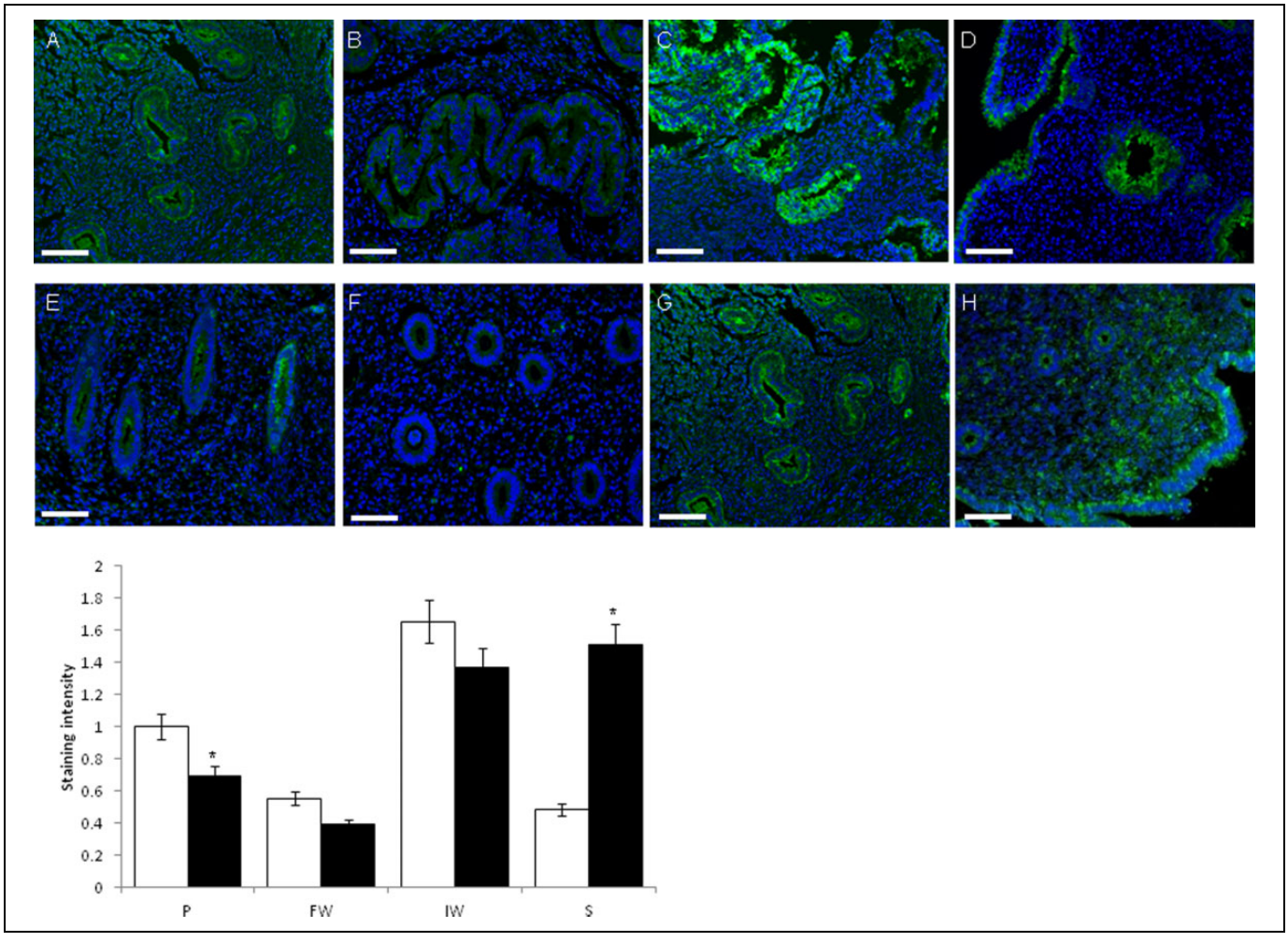

Figure 5. Top panel: Immunofluorescence staining of the GdA during menstrual cycle. Representative microphotographs of GdA staining in endometrium in proliferative phase (A and E), fertilization window (B and F), implantation window (C and G), and secretory phase (D and H) from healthy fertile women (A-D) and women with endometriosis (E-H). GdA, green; 4′,6-diamidino-2-phenylindole (DAPI), blue. Scale bar, 100 μm. Bottom panel: The intensity of GdA is shown in endometrium from women of proven fertility (white bars) and in endometrium of patients with endometriosis (black bars) during the menstrual cycle. The values are calculated in comparison with the proliferative phase of healthy endometrium (considered to be equal to 1). Data are represented as mean (standard deviation). $*P = 0.033$; $**P = 0.0042$. FW indicates fertilization window; GdA, glycodelin-A; IW, implantation window; P, proliferative phase; S, secretive phase.

Discussion

The endometrium is a highly dynamic tissue undergoing several biochemical and histological modifications under hormonal regulation during the menstrual cycle. The expression of GdA in normal human endometrium has been clearly demonstrated by means of a variety of techniques including immunohistochemistry and *in situ* hybridization.[14] However, little is still known about its expression during the different phases of the menstrual cycle and under pathological conditions, with somehow conflicting results. In the light of its potential importance in embryo implantation, GdA has been proposed to represent a potential biochemical marker of endometrial function. As a corollary, it can be assumed that its expression may be altered in pathological conditions. Here, we approached the study of GdA expression in the different phases of the menstrual cycle by semiquantitative immunoblotting analysis of endometrial tissue from fertile women and women affected by endometriosis. This allowed us to give an estimation not only of the overall GdA protein production but also of the GdA glycoform distributions in the different phases. Altogether, these results led to the conclusion that GdA expression changes significantly during the normal menstrual cycle, as well as its glycoform composition. In particular, GdA is present during the proliferative phase but, as expected, it almost completely disappeared at the fertilization window, whereas it reaches its maximum expression at the window of implantation, decreasing again during the late secretory phase. These data are essentially in agreement with those obtained by Mylonas and colleagues[14] by immunohistochemistry and in situ hybridization and by Kao et al[16] by global gene profiling.

Our data shed also light on GdA glycoform composition. The definition of glycoform is validated by the demonstration that after removal of N-linked oligosaccharide chains, a single

reactive spot can be observed after 1-D and 2-D electrophoresis and immunoblotting. This aspect is particularly important since the formation of different glycoforms and their biological activity is strictly due to the microheterogeneity of glycodelin 2 N-glycosylation sites. In addition, it should be noted that the high degree of sialylation and fucosylation is an important characteristic of GdA; sialic acid, in fact, by increasing the negative charge of the protein may affect the protein folding and function, as also demonstrated in patients with gestational diabetes mellitus, where the glycosylation level of GdA has clinical implication.[20] In fact, it has been suggested that the reduced sialylation observed in GdA from gestational diabetes may lead to reduced lymphocyte binding/immunosuppressive activity. Moreover, it has been demonstrated that steroid hormones may affect glycoprotein glycosylation, a process that has been showed to occur also during the normal human menstrual cycle and during pregnancy.[28] We demonstrated that in all analyzed endometrial samples, 4 to 5 glycoforms of GdA are usually present. A further point that deserves attention is the appearance, at the window of implantation, of a new acidic glycoform; whose isoelectrophoretic behavior may be due to increased sialylation. This selective expression suggests a crucial role of this glycoform in endometrial receptivity, confirming data indicating GdA as a key regulatory protein of the attachment between the embryo and the recipient endometrial epithelium.[29]

Glycodelin-A has also been proposed as a potential biomarker of endometriosis; indeed several studies reported an increase in GdA concentration in serum and peritoneal fluid of women affected by this pathology.[7,18,30] Moreover, by both immunohistochemistry and microarray analysis, it has been reported a decrease in GdA expression at the window of implantation in eutopic versus healthy endometrium.[5,19] Our results are partially in agreement with these papers since we could confirm, by 2-D electrophoresis and immunofluorescence analysis, a decrease in GdA expression at the window of implantation in eutopic compared to healthy endometrium. In addition, for the first time, by means of both techniques, we were able to demonstrate a statistically significant decrease in reactivity in eutopic endometrium during the proliferative phase and a statistically significant 10-fold increase in the late secretory phase. It is well known that GdA is under the regulation of progesterone[31]; even if women with endometriosis exhibit normal circulating ovarian progesterone levels, they have a reduced endometrium's ability to respond appropriately to this steroid.[32] Therefore, altered expression of genes and proteins reported in endometrial tissues from women with endometriosis has been associated with changes in the expression pattern of progesterone receptor isotypes (PR-A/PR-B), both in eutopic endometrium and ectopic lesions.[5,33] Hence, it may be hypothesized that GdA altered expression should be related to progesterone failure to properly direct the endometrial differentiation during each phase of the menstrual cycle.

In summary, our results suggest that GdA expression and the differential presence of its glycoforms are somehow altered in the endometrial epithelium of women affected by endometriosis being, in particular, strongly elevated during the late secretory phase. This may account for the fertility problems diagnosed in endometriotic women, suggesting that endometrial abnormalities may create an unreceptive endometrial environment, due to different mechanisms, ranging from inflammation and immune deregulation to apoptosis activation.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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