Glycodelin blocks progression to S phase and inhibits cell growth: a possible progesterone-induced regulator for endometrial epithelial cell growth

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Prolonged exposure to unopposed estrogen in the absence of progesterone gives rise to endometrial hyperplasia and carcinoma. Post-ovulatory progesterone is necessary for the proper growth and differentiation of endometrial epithelial cells (EECs). Progesterone exposure induces the endometrial production of numerous bioactive substances, one of which is the glycoprotein, glycodelin (Gd). We investigated the role of Gd in cell cycle progression and cell growth to better understand how Gd affects EEC behavior and endometrial cancer pathogenesis. Ishikawa cells, a well-differentiated human endometrial epithelial cancer cell line, were transfected with expression plasmids encoding enhanced green fluorescent protein (EGFP) or EGFP-fused Gd (EGFP-Gd). They were then subjected to a cell proliferation assay, flow cytometry cell cycle analysis and RT–PCR analysis of cyclin-dependent kinase inhibitors (CDKIs) including p21, p27 and p16. Overexpression of EGFP-Gd resulted in a reduction of cell proliferation activity, an accumulation of G1-phase cells and up-regulation of p21, p27 and p16 mRNAs. Furthermore, progesterone-induced inhibition of Ishikawa cell growth was partially attenuated by Gd knockdown using siRNA. These results indicate that Gd causes inhibition of G1/S progression together with up-regulation of CDKIs thereby reducing cell growth. Thus, progesterone-induced expression of Gd may, at least in part, contribute to the suppression of endometrial epithelial growth observed during the secretory phase.

Keywords: glycodelin; endometrium; cell cycle; cyclin-dependent kinase inhibitors; progesterone

Introduction

Glycodelin (Gd) is a secretory phase dominant glycoprotein that is synthesized by endometrial epithelial cells (EECs) in response to progesterone exposure (Seppälä et al., 2002). During the proliferative phase, the human endometrium contains no detectable Gd (Seppälä et al., 1988; Waites et al., 1988). However, it appears in some endometrial glands 4–5 days after ovulation, then gradually increases such that 10 days after ovulation all glands are strongly positive (Seppälä et al., 2002). Its temporal and spatial expressions have made Gd a widely used marker of endometrial differentiation (Seppälä et al., 2002). Gd inhibits oocyte–sperm binding in a dose-dependent manner (Oehninger et al., 1995). Additionally, it has an immunosuppressive effect, potentially inactivating T cells and natural killer cells (Okamoto et al., 1991). These observations suggest that Gd might contribute to contraceptive activity during the latter half of the secretory phase, and may also protect the embryonic semi-allograft from maternal immune insults. Aberrant expression of Gd has been reported in pathological conditions of the human endometrium and in endometrium-derived disorders including endometriosis (Seppälä et al., 2002; Taylor et al., 2002). Decreased immunostaining of Gd is associated with histologically retarded endometrium, suggesting that it protects against implantation failure (Klentzeris et al., 1994). Malignant endometrium does not appear to synthesize Gd (Wood et al., 1988), whereas serum Gd levels are elevated in patients with advanced endometriosis (Telimaa et al., 1989).

We have previously reported that Gd is involved in histone deacetylase inhibitor-enhanced cytodifferentiation and cell motility in Ishikawa cells. These cells are a well-differentiated human EEC line (Uchida et al., 2005, 2007). From these initial experiments, we noted that overexpression of Gd inhibited cell growth in Ishikawa cells. The objective of this study was to confirm this inhibitory effect of Gd on cell growth, elucidate its molecular basis and address the biological relevance of Gd in the behavior of EECs. We demonstrate here that Gd provokes G1 arrest with simultaneous up-regulation of genes that inhibit cell cycle progression and cell growth. In this manner, Gd behaves as an effector molecule for progesterone action.

Materials and methods

Reagents, plasmids and small interference RNA (siRNA)

Phenol red-free MEM and fetal bovine serum were purchased from Invitrogen Life Technologies (Tokyo, Japan). All oligonucleotides were synthesized by Invitrogen Life Technologies. Antibodies against Gd (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), green fluorescent protein (GFP)
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(BD Biosciences, Bedford, MA, USA), mitogen-activated protein kinases (MAPK) (Upstate Biotechnology, Inc., Lake Placid, NY, USA) and horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were purchased from commercial sources. Unless indicated otherwise, all other chemicals were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA) and Wako Biochemicals (Osaka, Japan). An expression plasmid harboring enhanced GFP (EGFP) or the EGFP-fused Gd gene (EGFP-Gd) and siRNAs for GAPDH and Gd were prepared as previously described (Uchida et al., 2005, 2007).

Cell culture

The human EEC line Ishikawa (clone 3-H-12) was kindly provided by Dr Masato Nishida (National Kasumigaura Hospital, Ibaraki, Japan). The Ishikawa cells were maintained in phenol-red free MEM supplemented with 10% charcoal-treated fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C under 5% CO₂ in a humidified incubator and washed and resuspended at 10⁷ cells/ml in media supplemented with 20 μg of Hoechst 33342, a cell-permeable DNA dye and incubated for 15 min at 37°C. The absorbance at 490 nm was measured using the Ultraspec Visible Plate Reader II 96 (Amersham Biosciences, Piscataway, NJ, USA).

For knockdown experiments, Ishikawa cells were transfected with or without GAPDH or Gd siRNA, and treated without or with 1 μM progesterone in combination with 10 nM 17β-estradiol (EP) for 48 h, and then subjected to the MTS assay.

RNA extraction and RT–PCR

Ishikawa cells were transfected with expression plasmids encoding EGFP or EGFP-Gd and cultured for 24 h before being harvested. EGFP-positive cells were then sorted by flow cytometry. Total RNA was extracted from the sorted cells using an RNA isolation kit (TAKARA, Tokyo, Japan). RT–PCR was carried out with 80 ng of total RNA using the One-Step RT–PCR kit (Qiagen, Hilden, Germany). The thermal cycling profile for p21, p27, p16 and GAPDH was 50°C for 30 min as a hot start time step, 94°C for 15 min as an initial denaturation step, 25 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, followed by a final extension of 72°C for 10 min. Forward (F) and reverse (R) primers used in this study were as follows: p21, 5'-GTCCGTCAAGAACCATGC-3' (F) and 5'-GGCGTTTGGATGTTAGAAA-3' (R); p27, 5'-AAATGTTCAGACGGTTCCC-3' (F) and 5'-ACAGAGATGTCATCCATTAGA-3' (R); p16, 5'-CCTCCTCCGGCCCGAT-3' (F) and 5'-CATGGTTACTGCCTCTGT-3' (R); and GAPDH, 5'-TACACATCTCGAGAGGGCG-3'(F) and 5'-CTGGTGACCAACCTCTGGA-3'(R). After PCR amplification, samples were electrophoresed in 2% agarose gels, followed by photographic recording of the ethidium bromide-stained gels with the FAS-III MINI (Toyobo, Tokyo, Japan). The band intensities were measured using Image J (version 1.38; http://rsb.info.nih.gov/ij/download.html). The relative ratio was calculated as the densitometry of each CDK1 divided by that of GAPDH, and the relative ratio of EGFP-expressing cells was set at 1.0. All experimental data for RT–PCR represent the results obtained from three independent experiments.

Immunoprecipitation and immunoblotting

The procedures were described previously (Uchida et al., 2005, 2007). In brief, Ishikawa cells transfected without or with siRNA were cultured for 3 days and lysed on ice with RIPA buffer (20 mM Tris–HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1% Na-deoxycholate; 0.1% sodium deoxycholate; 1 mM Na3VO4; 50 mM NaF; 1 mM Na2MoO4) containing protease inhibitor cocktail (Roche, Basel, Switzerland). Protein concentrations were determined using DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with BSA as a standard. Each 250 μg of protein was subjected to immunoprecipitation with anti-Gd antibody and protein G sepharose beads (Amersham Biosciences) for 3 h at 4°C. Immunoprecipitates were separated by electrophoresis on a 12% SDS–PAGE gel and transferred onto polyvinylidene difluoride membrane. After incubation with anti-Gd antibody, followed by horse-radish peroxidase-conjugated secondary antibody, the immunoreactive proteins were detected by the enhanced chemiluminescence method (Amersham Biosciences). Each input cell lysate (10 μg) was also subjected to immunoblotting with anti-MAPK antibody.

Cell proliferation assay (MTS assay)

Ishikawa cells were transfected with expression plasmids encoding EGFP or EGFP-Gd using LipofectAMINE2000 (Invitrogen Life Technologies), and sorted by flow cytometry based on the intensity of EGFP fluorescence 24 h later. The sorted cells (7 × 10⁵ cells) were re-plated onto 96-well plates, grown for an additional 24 or 48 h and subjected to the Cell Titer 96 AQueous One Solution Cell Proliferation Assay (Promega Corp., Madison, WI, USA) using MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2H-tetrazolium) according to the manufacturer’s protocol. In brief, 20 μl of Cell Titer 96 AQueous One Solution Reagent was added into each well of the 96-well assay plate containing the samples in 100 μl of culture medium. The plate was incubated for 1.5 h at 37°C in a humidified, 5% CO₂ atmosphere. The absorbance at 490 nm was measured using the Ultrospec Visible Plate Reader II 96 (Amersham Biosciences, Piscataway, NJ, USA).

Cell cycle analysis by fluorescence-activated cell sorting and flow cytometry

The DNA distribution profiles of the EGFP- or EGFP-Gd-expressing cells were determined by a combination of cell sorting and flow cytometry using the EPICS ALTRA (Beckman coulter, Fullerton, CA, USA). Cultures transfected with expression plasmids encoding EGFP or EGFP-Gd were harvested, washed and resuspended at 1 × 10⁴ cells/ml in media supplemented with 20 μg of Hoechst 33342, a cell-permeable DNA dye and incubated for 45 min at 37°C. Cell suspensions were then filtered through a 5 ml Polystyrene Round-Bottom Tube with a Cell-Strainer Cap (BD Falcon, Bedford, MA, USA), and further incubated at room temperature with 5 μg/ml propidium iodide (PI), a cell-impermeable DNA dye, for 5 min. The cells were sorted with the cytometer’s Argon I-90 laser set at 488 nm in the primary position for EGFP and the Krypton laser 300 series tuned to a multline ultraviolet spectrum in the secondary position for Hoechst 33342. The EGFP signal was collected through a 450/50-band pass filter. The PI signal was excited with the Argon laser and collected through a 660/22-band pass filter. Both lasers were set with 100 mW of light. Each sample was sorted, and data were collected (10,000 events) using the EPICS ALTRA. Final analysis of the collected data, notably the DNA content histograms, was done using the software program MultiCycle (Beckman coulter). PI-positive cells and doublet were excluded to ensure that only single viable cells were subjected to the analysis of DNA content and EGFP expression.

Statistical analysis

All statistical analyses were performed with the software package JMP version 6.0 (SAS Institute Inc., Cary, NC, USA). Data were analyzed by the Wilcoxon rank sum test, unpaired t-test or Dunnett’s test following ANOVA. P < 0.05 was considered significant.

Results

Inhibition of cell growth by Gd

Gd expression is nearly undetectable in Ishikawa cells (Arnold et al., 2002; Uchida et al., 2005) and EECs of the proliferative phase (Seppälä et al., 2002). Upon exposure to progestins and/or histone deacetylase inhibitors, however, Gd expression is induced in Ishikawa cells (Arnold et al., 2002; Uchida et al., 2005, 2007) as well as in EECs of the secretory phase (Seppälä et al., 2002). We began this study with experiments in which Gd was overexpressed in order to investigate its role in EEC function. We transfected Ishikawa cells with EGFP alone or EGFP-Gd to assess the effect of Gd on cell growth. The transfected cells (2 × 10⁵ cells) were plated, cultured and harvested for counting cell number. Both types of transfected cells exhibited similar increases in number at 24 h after transfection; however, at 48 h, cells transfected...
with EGFP-Gd had significant inhibition of cell growth when compared with cells transfected with EGFP alone (Fig. 1).

Although simple transfection with EGFP-Gd significantly inhibited cell growth, the reduction rate was only ~30% (Fig. 1). A larger reduction was not seen partly because of the relatively low (~20%) transfection efficiency of EGFP-Gd. To minimize the influence of untransfected cells, EGFP-positive Ishikawa cells were sorted by flow cytometry 24 h after transfection of EGFP or EGFP-Gd, based on the intensity of EGFP fluorescence (Fig. 2A). The cells obtained from sorting following transfection of the different plasmids were designated as EGFP- or EGFP-Gd-expressing cells. They were then plated, cultured and harvested 48 and 72 h after transfection, and subjected to the MTS assay. The EGFP-Gd-expressing cells exhibited a significant reduction (50%) in proliferation activity (Fig. 2B).

**Inhibition of G1 to S cell cycle progression by Gd**

To examine whether the retardation of cell growth was due to the inhibition of cell cycle progression, we analyzed the cell cycle distribution of purified Ishikawa cells overexpressing EGFP or EGFP-Gd. Flow cytometric analysis revealed that EGFP-Gd-expressing cells accumulated in the G1 phase of the cell cycle, with a concomitant decrease in the proportion of those in the S and G2/M phases, as compared to EGFP-expressing cells (Figs. 3A and B). These changes in cell cycle distribution were observed at 24 h after transfection and became more evident and statistically significant at 48 h (Fig. 3B).

To determine whether the inhibitory effect of Gd on cell growth was dose-dependent, we compared the cell cycle distribution between transfected Ishikawa cells with high and low levels of Gd expression. As shown in Fig. 3C, EGFP-positive cells were further gated based on the intensity of EGFP fluorescence and were divided into two distinct populations: high and low. Cell cycle analysis of each gated population revealed that high EGFP-Gd-expressing cells significantly accumulated in the G1 phase with a concomitant decrease in the proportion in S phase (Fig. 3D). The patterns of cell cycle distribution were similar among the other three populations (Fig. 3D).

**Up-regulation of cyclin-dependent kinase inhibitors by Gd**

Cyclin-dependent kinase (CDK) inhibitors including p21, p27 and p16 are well-known negative regulators of the G1/S transition (Shapiro, 2006). Overexpression and/or induction of these CDK inhibitors (CDKIs) lead to G0/G1 accumulation (Shapiro, 2006). To address a possible involvement of CDKIs in the inhibition of the G1/S transition by Gd, we examined the expression of p21, p27 and p16 in EGFP- or EGFP-Gd-expressing cells by RT–PCR. EGFP-Gd significantly up-regulated the levels of p21, p27 and p16 mRNAs 48 h after transfection (Fig. 4). GAPDH, an internal control gene, was constant among each group of transfected and sorted cells (Fig. 4).

**Attenuation of progesterone-induced inhibition of Ishikawa cell growth by Gd knockdown using siRNA**

Progesterone inhibits cell growth in Ishikawa cells while simultaneously up-regulating Gd (Uchida et al., 2005, 2007). This raises the possibility that progesterone-induced Gd may be involved in the inhibition of EEC cell growth. To test this, Ishikawa cells were transfected without or with siRNA for GAPDH or Gd, and treated without or with EP for 48 h, prior to being used in the MTS assay.
In agreement with previous studies (Uchida et al., 2005, 2007), treatment with EP-induced Gd expression, and the induction of Gd by EP treatment, is largely repressed by Gd siRNA, but not GAPDH siRNA (Fig. 5A). Furthermore, we showed that treatment with EP significantly inhibited cell growth (Fig. 5B). Importantly, the inhibitory effect was significantly abrogated by Gd siRNA, but not by GAPDH siRNA (Fig. 5B).

**Discussion**

CDK is a protein kinase involved in regulating the cell cycle. It is activated by associating with a cyclin, forming a CDK complex. CDKIs, including p21, p27 and p16, are a group of proteins that interact with and inhibit the CDK complex. This negatively affects cell cycle progression thereby retarding cell growth (Shapiro, 2006). In the present study, overexpression of Gd alone inhibited the G1/S transition and cell growth, and also increased expression of p21, p27 and p16. Thus, Gd-induced G1 arrest may be, at least in part, attributable to the up-regulation of CDKIs by Gd.

Gd appears in some endometrial glands 4–5 days after ovulation, then gradually increases such that at 10 days after ovulation all glands are strongly positive (Seppälä et al., 2002). The induction of endometrial Gd is dominantly regulated by progesterone (Seppälä et al., 2002). Ishikawa is a well-differentiated endometrial cancer cell line of human glandular epithelial origin that expresses functioning estrogen receptor α and the progesterone receptor (PR). These qualities have led to its widespread use for studies of human EEC pathophysiology (Nishida, 2002). Indeed, Ishikawa cells possess similar properties to normal EECs (Nishida, 2002), including the ability to express Gd in response to progesterone (Uchida et al., 2005). A close association between progesterone-induced Gd expression and cell growth inhibition was evident in our study. This suggests a possible mechanism by which progesterone-induced Gd may inhibit cell growth of EECs during the late secretory phase. In agreement, several studies have reported that Ki-67, a cell proliferation marker, is down-regulated, whereas p21 and p27 are
Up-regulation of CDKIs in EECs during the secretory phase (Shiozawa et al., 1998; Toki et al., 2000). Notably, although Gd expression is positively associated with a good prognosis (Mandelin et al., 2003; Shabani et al., 2005). Chemotherapy-treated patients with Gd-expressing serous ovarian carcinoma have longer survival times than those with Gd-negative tumors with the same differentiation grade and clinical stage (Mandelin et al., 2003). Furthermore, patients with Gd-positive breast tumors have a better prognosis when compared with patients with Gd-negative tumors (Mandelin et al., 2003; Shabani et al., 2005). The improved prognosis may be the result of a combination of Gd actions including induction of cytodifferentiation (Kämäräinen et al., 1997; Uchida et al., 2005) and apoptosis (Koistinen et al., 2005), as well as its anti-proliferative activity (Kämäräinen et al., 1997; Koistinen et al., 2005). In this regard, Gd may be useful not only as a marker but also as a targeting molecule in the management and treatment of endometrial cancer and other endometrium-derived diseases.

In summary, Gd causes inhibition of G1/S progression together with up-regulation of CDKIs, thereby reducing cell growth. Gd may be, at least in part, responsible for the progesterone-mediated inhibition of cell growth. Thus, progesterone-induced expression of Gd during the secretory phase may contribute to the suppression of endometrial epithelial growth. Gd may act as an inducible effector molecule of progesterone action, possibly regulating the growth and differentiation of EECs during the menstrual cycle. In controlling endometrial growth, it may prevent the progression of endometrium-derived diseases including endometrial cancer.

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References


Figure 5: Attenuation of progesterone-induced inhibition of Ishikawa cell growth by Gd siRNA

(A) Ishikawa cells were untransfected (none) or transfected with GAPDH siRNA or Gd siRNA, treated without or with EP for 3 days and harvested. Total cell lysates were extracted and subjected to immunoprecipitation and immunoblotting with anti-Gd antibody (upper panels). Each input cell lysate was subjected to immunoblotting with anti-MAPK antibody (lower panels). (B) Ishikawa cells were untransfected (none) or transfected with GAPDH siRNA or Gd siRNA, treated without (EP−, white bar) or with EP (EP+, gray bar) for 48h and then subjected to the MTS assay. Bars indicate the mean ± SD of the relative MTS ratio obtained from four independent experiments. MTS value of EP−untreated cells without siRNA transfection was set at 1.0. *P < 0.01; **P < 0.05; n.s., not significant (ANOVA and Dunnett’s test).

up-regulated in EECs during the secretory phase (Shiozawa et al., 1998; Toki et al., 1998; Bebington et al., 2000). Notably, although Gd siRNA abrogated progesterone-induced Gd expression (Uchida et al., 2005, 2007), it only partially rescued the inhibition of Ishikawa cell growth. This suggests that progesterone-regulated gene product(s) other than Gd may also contribute to the inhibitory effects. Given the up-regulation of CDKIs by Gd overexpression, Gd may be, at least in part, involved in the inhibition of cell growth together with up-regulation of CDKIs in EECs during the secretory phase.

The PR activates the natural promoters for p21, p27, and p16 and Gd (Tung et al., 1993; Owen et al., 1998; Gao et al., 2001; Smid-Koopman et al., 2003; Gizard et al., 2005). In particular, p21, p27 and Gd promoters are stimulated by the PR through G/C-rich (Sp1 binding) elements, but not through the functional classical response element (Tung et al., 1993; Owen et al., 1998; Gao et al., 2001; Gizard et al., 2005). Although it is unknown whether Sp1 is involved in PR-induced up-regulation of p16, Sp1 is required for the activation of the p16 promoter in human fibroblasts (Wu et al., 2007). We demonstrated that overexpression of Gd alone resulted in the induction of p21, p27 and p16 in the absence of progesterone. These findings raise the possibility that the PR induces Gd, which, in turn, may augment the induction of the CDKIs, by PR, through Sp1 sites. In this regard, PR-induced Gd may act inside the cell, since Ishikawa cells are not capable of secreting Gd (Arnold et al., 2002). Although the function of intracellular Gd is poorly understood, it has been reported to mediate the histone deacetylase inhibitor-driven induction of leukemia inhibitory factor (Uchida et al., 2005). Additionally, it also regulates the expression of several genes including MUC1, vimentin, E-cadherin and cytokeratins 8 and 18 (Kämäräinen et al., 1997; Koistinen et al., 2005).

Gd has been observed in various benign and malignant tumors (Seppälä et al., 2002). Intriguingly, Gd expression in cancer is positively associated with a good prognosis (Mandelin et al., 2003; Shabani et al., 2005). Chemotherapy-treated patients with Gd-expressing serous ovarian carcinoma have longer survival times than those with Gd-negative tumors with the same differentiation grade and clinical stage (Mandelin et al., 2003). Furthermore, patients with Gd-positive breast tumors have a better prognosis when compared with patients with Gd-negative tumors (Mandelin et al., 2003; Shabani et al., 2005). The improved prognosis may be the result of a combination of Gd actions including induction of cytodifferentiation (Kämäräinen et al., 1997; Uchida et al., 2005) and apoptosis (Koistinen et al., 2005), as well as its anti-proliferative activity (Kämäräinen et al., 1997; Koistinen et al., 2005). In this regard, Gd may be useful not only as a marker but also as a targeting molecule in the management and treatment of endometrial cancer and other endometrium-derived diseases.


