Relaxin stimulates glycodelin mRNA and protein concentrations in human endometrial glandular epithelial cells

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Human endometrium is the major organ that produces glycodelin A (GdA). The production of endometrial GdA causes a fluctuation of the peripheral glycodelin concentrations in women during the menstrual cycle and pregnancy. It has recently been reported that the rise of plasma concentrations of glycodelin is correlated with relaxin during the late luteal phase and early pregnancy. In addition, administration of relaxin increases glycodelin plasma concentrations, suggesting that relaxin induces GdA production in endometrium. To investigate whether relaxin regulates the GdA synthesis, human endometrial glandular epithelial cells were isolated and cultured with or without relaxin for up to 4 days. Western blot showed that GdA synthesized and secreted from epithelial glands had a major molecular weight of 28 kDa, i.e. the same as the GdA isolated from amniotic fluid. Cells incubated with relaxin consistently increased in GdA production rate (2–6-fold). The GdA mRNA concentrations increased 2–11-fold in cells incubated with relaxin for 2–4 days, as determined by solution hybridization/ribonuclease protection assay. The increase of the mRNA concentration indicates that relaxin activates GdA transcription.

Key words: endometrium/glycodelin/relaxin

Introduction

Human endometrium and early gestational decidua secrete glycodelin A (GdA) which was previously known as placental protein-14, PP14. In the normal ovulatory cycle, GdA secretion begins 4–5 days after ovulation and is maintained until the next menstrual cycle (Julkunen et al., 1986; Wood et al., 1989; Fay et al., 1990). After conception, the production rate of GdA continues to increase in the decidua of early gestation (Julkunen, 1986; Bell et al., 1988). GdA synthesis and production in endometrium possibly facilitate the process of implantation and maintains pregnancy since GdA has immunosuppressive activity (Dell et al., 1995; Seppälä et al., 1997). In addition to its immunosuppressive activity, GdA contains unique carbohydrate sequences, potentially inhibits the initial binding of spermatozoa to the zona pellucida (Dell et al., 1995; Oehninger et al., 1995). Thus, the temporal expression of GdA in endometrial glands is likely to be critical for fertility, as absence of GdA before and at the time of ovulation appears to be a prerequisite for fertilization (Seppälä et al., 1997). However, little is known of the regulation of endometrial GdA production.

Glycodelin gene activation has been associated with progesterone since only the progesterone-sensitized endometrium in the luteal phase increases the glycodelin secretion (Joshi 1983; Julkunen et al., 1986). Progesterone treatment in the luteal phase of ovulatory infertile women increases the serum glycodelin concentration (Seppälä et al., 1987). However, mean serum glycodelin concentrations were not correlated with the serum progesterone concentrations (Fay et al., 1990). During the first trimester, the serum concentrations of glycodelin increased from 10 to 350 ng/ml from 2–10 weeks of gestation and the amniotic fluid GdA accumulated up to 230 µg/ml at 10–16 weeks of gestation (Julkunen, 1986). It is likely that progesterone exerts an indirect effect and hormones produced during pregnancy, such as relaxin (RLX) or human chorionic gonadotrophin (HCG), play essential roles on the production of endometrial GdA.

A recent study has shown that RLX given to women with a normal menstrual cycle increases their circulating glycodelin concentrations and that progesterone was noted to be unlikely as a stimulus compared with RLX (Stewart et al., 1997). These observations strongly suggest that RLX induces endometrial GdA production.

In order to clarify the role of RLX and progesterone, we have investigated their effects on GdA in the human endometrial glandular epithelial cells in culture. Our results demonstrate that RLX induced GdA production and its mRNA content in endometrial glands. In addition, we have shown that RLX stimulates the cAMP accumulation in endometrial glandular cells (Chen et al., 1988). Thus, we have also examined the effect of a phosphodiesterase inhibitor on the content of GdA mRNA in glandular cells.

Materials and methods

Tissue and cell culture

Human endometrial specimens (n = 10) were obtained from premenopausal women who had undergone hysterectomy for medical
reasons such as uterine prolapse, fibroid uterus, and adenomyosis, but not endometrial hyperplasia and endometrial cancer. Endometrial specimens were diagnosed histologically and classified as proliferative, early, mid- and late secretory endometrium. Use of these human specimens was approved by the Human Subjects Committee of our institution in accordance with US Department of Health regulations.

To isolate endometrial glandular cells, tissue fragments were repeatedly digested with collagenase in Hank’s balanced salt solution (~0.5 g tissue/5 ml) for 10–15 min to remove stromal cells (Liu and Tseng, 1979; Tseng and Zhu, 1998). Glands were sedimented and further purified by an additional collagenase digestion (30 min) and repeatedly suspended to remove detached stromal cells. Intact and fragmented glands were treated with trypsin (3–4 min), centrifuged and suspended in medium A (Roswell Park Memorial Institute 1640 medium, 100 IU/ml penicillin, 100 µg/ml streptomycin) with 2% fetal bovine serum (FBS). Any trace amount of stromal cells were removed by sequential attachment in which stromal cells attach more quickly to the plastic surface (within 2 h), leaving the glands in suspension. The glands were then transferred to Petri dish precoated with 1.5% gelatin (BioRad, Hercules, CA, USA). The purity of the cultured glandular cells was accessed to be >98% by the uniform staining of keratin (Tseng and Zhu, 1998).

To study the effects of hormones and other agents on GdA production and mRNA values, glandular cells were incubated with progesterone (0.1 µmol/l), medroxyprogesterone acetate (MPA 0.1 µmol/l; Steraloids Inc, Wilton, NH, USA), or porcine RLX (100 ng/ml; a gift from Dr Sherwood, University of Illinois), or a phosphodiesterase inhibitor, Ro [50 µmol/l; (4-(3-butoxybenzyl)-2-imidazolidinone; Hoffman La Roche Inc, Nutley, NJ, USA] for various lengths of time, as specified in each experiment. The concentrations of the various compounds used in this study were adapted from studies reported in previous publications from our laboratory. The biological activity of 1 mg RLX is equivalent to 3000 IU of mouse pubic symphysis (Sherwood, 1974). We have used porcine RLX since it is equally potent to the human recombinant RLX (unpublished observation). Cells were incubated with hormones for 1–4 days as indicated in each experiment. Media and cell lysates were separately collected to determine the GdA content.

Western blot and determination of GdA

To characterize the endometrial GdA, cells were lysed in a 1% sodium dodecyl sulphate (SDS), 0.01 M Tris–HCl (pH 7.4) solution. Samples of cell lysate (10 µg protein/lane) and medium were resolved by 12% SDS–polyacrylamide gel electrophoresis (PAGE) and subsequently blotted on a polyvinylidene difluoride (PVDF) membrane (Bio Rad) and stained of keratin (Tseng and Zhu, 1998). Western blot and determination of GdA activity of 1 mg RLX is equivalent to 3000 IU of mouse pubic symphysis (Sherwood, 1974). To study the effects of RLX on GdA secretion, cells were incubated with or without 100 ng/ml RLX for 1 day. The culture medium was replaced by serum-free medium and culture was continued for 2 h. Cell lysate (10 µg protein/lane) and aliquots of serum free media (equivalent to 10 µg cell lysate) in the control and RLX-treated samples were resolved on a 12% SDS–PAGE. The relative intensities of the 28 kDa band were scanned on Bio Rad Image analysis system using Molecular Analyst/PC software (Molecular Science, Chicago, IL, USA).

The amounts of GdA in cultured medium and in cell lysates were determined by a two antibody sandwich solid phase immunofluorometric assay (IFMA, Koistinen et al., 1996).

Determination of GdA mRNA by solution hybridization/ ribonuclease protection assay

Total RNA was isolated from glandular cells by a single-step acid guanidium thiocyanate extraction (Tseng et al., 1992). To synthesize an antisense probe of GdA mRNA, the SacI-RsaI fragment (+130 to +459 bp) of the pp14 (GdA cDNA (Julkunen et al., 1990) was ligated to pSP73. The orientation and sequence of the subclone were confirmed by DNA sequencing. The antisense [32P]-riboprobe was transcribed by the linearized pSP73-GdA, using an in-vitro transcription labelling kit (B&M Corporation, Minneapolis, MN, USA) described previously (Tseng et al., 1992). [32P]-UTP (S.A. ~800 Ci/mmol; Amersham Life Science, Chicago, IL, USA) and unlabelled ATP, CTP and GTP were used as substrates. An antisense 18s rRNA probe was synthesized in a similar manner using [32P]-UTP (S.A. 20 mCi/mmol) to identify 18s rRNA which serves as an internal standard. 5 µg of glandular RNA was hybridized with 100 000 cpm of [32P]-GdA and 500–2000 cpm 18s RNA probes using a ribonucleotide protein assay kit (RPAII; Ambion, Austin, TX, USA). After nuclease digestion, the SacI-RsaI fragments were resolved by gel electrophoresis (5% polyacrylamide/8M urea) and visualized by autoradiography. The relative intensities of the protected fragments were estimated by scanning the x-ray film using a Bio Rad Image Analysis System (Bio Rad).

Results

Glycodelin produced from glandular epithelial cells was analysed by Western blot using a purified polyclonal antibody to GdA (Koistinen et al., 1996). A major band of ~28 kDa was shown on the Western blot (Figure 1) identical to the GdA purified from the amniotic fluid in parallel (the right lane in Figure 1).

Figure 1 also showed that RLX stimulated GdA production in endometrial glandular cells. After 1 day of incubation with RLX, GdA increased 4.5-fold in medium accumulated in a 2 h period and 1.4-fold in cell lysate respectively. Glycodelin content in medium was also determined by IFMA. The basal secretion rate (cells cultured without hormone for 3 days) varied ranging from 17, 11, 100 and 15 ng GdA/10⁶ cells/day examined in four separate specimens (glands isolated from early, mid- and late secretory endometrium, and a 10 week of gestational decidual respectively), suggesting a correlation with the stage of menstrual cycle. In each experiment, cells incubated with RLX consistently increased the production rate of GdA by 2–6-fold in four specimens. The time course on the effect of RLX on the production of GdA was examined in one of the right lane.
The effect of relaxin (RLX) on the production rate of glycodelin (GdA) in human endometrial glandular cells. Glandular cells were isolated from an early secretory endometrium incubated with 100 ng/ml of RLX. Culture media were collected after 24, 48 and 72 h of incubation. Cell lysates were collected at the end of 72 h of incubation. Data represent mean of duplicated dishes ± range.

the specimens shown in Figure 2. The production rate in control cells, showed an increase of GdA from 17 to 100 ng/10⁶ cells/d from day 1 to day 3 of incubation suggesting a spontaneous increase under the culture condition, perhaps by serum factor(s) since cells were cultured in 2% FBS. The production rates increased 2, 8, and 6-fold in cells incubated with RLX for 1, 2 and 3 days respectively.

GdA mRNA content in glandular cells was analysed by RPAII. When the total RNA of glandular cells was hybridized with the [³²P]-RNA probe of GdA, a protected band with the size of 330 bp was observed. RLX increased the content of GdA mRNA 2–11-fold in glandular cells from eight endometrial specimens and decidual explants. Figure 3 shows the effect of RLX in three out of the eight specimens. In experiment 1, GdA mRNA increased 1.5- and 2.5-fold in cells incubated with Ro and Ro plus RLX. In experiments 2 and 3, RLX increased GdA mRNA content, 3- and 11-fold compared with that of control cells. In contrast, a lesser effect (1.4- and 1.8-fold) was observed when cells were incubated with progestin.

Figure 3. Autoradiographs of human endometrial glandular glycodelin (GdA) mRNA determined by ribonuclease protection assay kit (RPAII). Glandular cells isolated from three specimens: no. 1 (late proliferative phase); no. 2 and no. 3 (mid-secretory phase). Cells were cultured with or without relaxin (RLX; 100 ng/ml), progestosterone (P) or medroxyprogesterone acetate (MPA; 0.1 µM) for 3, 3 and 4 days for specimen nos. 1, 2 and 3 respectively. 50 µM Ro was used in specimen no. 1. Total RNA (5 µg) isolated from each sample was hybridized with the probe, resolved on the urea–polyacrylamide gel electrophoresis, and visualized by autoradiography.

Discussion

The present study demonstrated that RLX stimulates GdA production in human endometrial glandular cells. The data are in agreement with the in-vivo findings, i.e. the plasma concentration of GdA is correlated with the rise of RLX but not with the concentration of progesterone during the luteal phase (Stewart et al., 1997). An increase in GdA production in situ in endometrial glandular cells is likely to influence the differentiation of endometrium since expression of GdA alters the growth pattern and promotes differentiation in MCF-7 cells (Kamarainen et al., 1997).

In addition, we have demonstrated for the first time that RLX increases GdA mRNA in glandular cells suggesting that RLX stimulates GdA gene transcription or post-transcriptional process. The increase of GdA by Ro or RLX/Ro correlates with the increase in cAMP accumulation in glandular cells determined previously (Chen et al., 1988) suggesting that cAMP signal transduction cascade may be involved in the induction of GdA gene. The RPAII data (Figure 3) showed a major band with a size of 330 bp and two minor bands (<300 bp). The minor bands may be caused by degradation of the primary transcript. Alternatively, it suggests multiple forms of GdA mRNA which appears to be present in male genital tract (Koistinen et al., 1997). However, in endometrial cells, it is unlikely that alternate splicing would be active since a single size of GdA mRNA was observed by Northern blot (Julkunen et al., 1990).

Induction of GdA has been associated with progesterone since glycodelin concentration is low in the endometrium and peripheral blood in the proliferative phase (Julkunen et al., 1985, 1986). Our data indicated that 3–4 days incubation of progestogen slightly increased the mRNA values indicating that the effect of progesterin is slow and its effect is likely to be indirect, e.g. induction of local inducers such as RLX or transcription factors which control the promoter activity of the GdA gene. Hausermann et al. (1998) reported that oestrogen and progesterone alone did not stimulate GdA expression but up-regulated it in the presence of exogenous HCG in the...
baboon uterus, suggesting that the effect of progesterone on GdA expression involves multiple factors, rather than a simple pathway. Further study is necessary to elucidate the induction mechanism.

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References


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