Expression of progesterone receptor mRNA in the endometrium during the normal menstrual cycle and in Norplant® users

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The expression of endometrial progesterone receptor mRNA during the human menstrual cycle and in Norplant users was studied using digoxigenin-labelled ribonucleic probes for in-situ hybridization on 6 μm paraffin embedded endometrial sections. The staining intensity was scored blind semi-quantitatively. Blood ovarian steroid concentrations were measured in Norplant users. All data were analysed by analysis of variance. Glandular progesterone receptor mRNA concentrations were low during the menstrual-to-early proliferative stage but increased during the early-to-mid to late-proliferative stage then declined nonsignificantly over the secretory stage. No such variation was observed in stromal cells. Progesterone receptor mRNA concentrations were lower in Norplant than controls during early-to-mid to late-proliferative stages (in glandular epithelium and stroma) and during secretory stage (in stroma only). Norplant subjects with amenorrhoea had higher concentrations of stromal progesterone receptor mRNA but lower plasma oestrogen concentrations than subjects with breakthrough bleeding. The pattern of variation in progesterone receptor mRNA concentrations during the normal menstrual cycle resembles the published pattern for the receptor protein. The results demonstrate: (i) a differential sensitivity of glandular and stromal progesterone receptors to steroid regulation; (ii) in contrast to previous findings of an increase in immunoreactive progesterone receptor protein in Norplant endometrium, progesterone receptor mRNA concentrations in these tissues were reduced; and (iii) there was significantly more progesterone receptor mRNA in subjects with amenorrhoea than in those with breakthrough bleeding.

Key words: endometrium/in-situ hybridization/Norplant/progesterone/receptor

Introduction

During the normal menstrual cycle, the concentration of progesterone receptor protein in the human endometrium varies according to stage of the menstrual cycle and with different tissue compartments. The receptor protein concentrations in the glandular epithelium are low during the menstrual phase, increasing rapidly during the proliferative phase to a peak value in late proliferative to early secretory phase and then decline rapidly during the mid to late secretory phase (Press et al., 1988; Critchley et al., 1993). In contrast, progesterone receptor concentrations in the stroma remain relatively constant during the menstrual cycle (Press et al., 1988; Critchley et al., 1993).

In women using the levonorgestrel subdermal implants (Norplant®; Leiras Pharmaceuticals, Turku, Finland) as a contraceptive, plasma concentrations of the progestogen levonorgestrel are constantly elevated. During the first year of use, levonorgestrel is released from the implants at ~0.05–0.08 mg per day (Robertson et al., 1983) and peripheral plasma levonorgestrel concentrations remain steady at around 1.2 nM (Affandi et al., 1987; Olsson et al., 1987). Despite peripheral levonorgestrel concentrations being constantly elevated, immunoreactive progesterone receptor concentrations remain high in the endometrium of Norplant users (Critchley et al., 1993). This contradicts a previous report that progesterone down-regulates its own receptors (Nardulli and Katzenellenbogen, 1988).

The distribution and concentrations of progesterone receptor mRNA in the human endometrium during the normal menstrual cycle and in Norplant users are not known. The aim of this study was to determine the expression of progesterone receptor mRNA in the endometrium during the menstrual cycle and in Norplant users, using digoxigenin-labelled ribonucleic probes for in-situ hybridization.

Materials and methods

Subjects

Endometrial biopsies were taken from 53 Australian women across the normal menstrual cycle and from 39 Indonesian Norplant users. All human subjects were recruited on the basis of fully informed consent. Ethical approval was obtained from Monash University standing committee on ethics in research on humans and the medical faculty of the University of Indonesia ethics commission in research on humans. The biopsies were collected by using either a Pipelle suction curette (Prodimed 60530, Neuly-en-Thelle, France, control samples), Karman cannular (Rocket, London, UK; Norplant samples) or a microhysteroscope (4 mm, Karl-Storz, Tuttingen, Germany, both control and Norplant samples). Biopsies from the normal menstrual cycle were dated by an experienced histopathologist and according to stage of the menstrual cycle and with different tissue compartments.
buffer. The sections were fixed in 4% paraformaldehyde in KPBS (2630 O, 1.8 mM KH2PO4, 150 mM KCl (10 μg/ml) digestion was carried out at 37°C for 30 min in TEC buffer. The sections were washed in four changes of 100, 100, 75 and 30% ethanol. The Norplant biopsies were shipped to Monash University, Australia while stored in PBS.

A total of six blood samples was collected from each Norplant subject, with samples being taken every 2–3 days over 2 weeks prior to biopsy for oestradiol and progesterone evaluation. Both serum progesterone and oestradiol were measured using radioimmunoassay kits (Coat-A-Count Estradiol and Progesterone, Diagnostic Products Corporation, Los Angeles, CA, USA) (Crichtley et al., 1993). The inter-and intra-assay coefficients of variation for the oestradiol RIA were 5.5 (at 969 pmol/l) and 5.6% (at 1073 pmol/l) respectively. The inter-and intra-assay coefficients of variation for the progesterone radioimmunoassay were 7.2 (at 47.1 nmol/l) and 5.8% (at 57.2 nmol/l) respectively.

**RNA probes**

The human progesterone receptor cDNA clone was kindly donated by Professor P. Chambon (INSERM, Strasbourg Cedex, France). The cDNA was subcloned into the EcoRI and Hind III sites of a Bluescript II KS+ vector (Stratagene, La Jolly, CA, USA). DNA sequencing confirmed that clone PRBS5 contains the insert which covers the nucleotide sequence 273-1679 in the A/B region (Kastner et al., 1990) of the progesterone receptor cDNA (sequence name: HSPGRR, PC Gene). Sense and antisense digoxigenin (DIG)-labelled RNA-probes were transcribed using 1 μg of the linearized PRBS5 clone with T3 and T7 promoters respectively. All materials for the transcription reactions were purchased from Boehringer Mannheim (Mannheim, Germany) and the transcription reactions were performed according to the manufacturer’s instructions. At the end of the transcription, the template DNA was digested by adding 10 units of RNase-free DNase I and incubating at 37°C for 15 min. The sense and antisense probes were purified by ethanol precipitation. Alkaline hydrolysis was carried out in carbonate buffer (80 mM NaHCO3, 120 mM Na2CO3, 20 mM β-mercaptoethanol, pH 10.0) for 16 min at 60°C to yield an average probe length of 400 base pairs. The sense and antisense probes were further purified by ethanol precipitation. The concentrations of the sense and antisense probes were determined by spectrophotometry and a dot blot assay.

**In-situ hybridization**

Sections (6 μm) of endometrial biopsies were cut and attached to pre-cleaned 3-aminopropyl-triethoxy silane (Sigma, St Louis, MO, USA)–coated microscope slides. The sections were dehydrated and washed in four changes of 100, 100, 75 and 30% ethanol. The sections were rehydrated using sterilized reverse osmosis (RO) water and then treated with 0.2 N HCl for 5 min at room temperature followed by rinses with sterilized RO water and TEC buffer (100 mM Tris/HCl, 50 mM EDTA and 2 mM CaCl2, pH 7.4). Proteinase K (10 μg/ml) digestion was carried out at 37°C for 30 min in TEC buffer. The sections were fixed in 4% paraformaldehyde in KPB (3.5 mM KCl, 4 mM Na2HPO4,12H2O, 1.8 mM KH2PO4, 150 mM NaCl, pH 7.4) at 4°C for 3 min followed by rinses with sterilized RO water and triethanolamine buffer (10 mM, pH 8.0). Acetylation was carried out in 0.25% acetic anhydride in triethanolamine buffer for 10 min at room temperature. Prehybridization was carried out at 48–50°C for 2 h using 50 μl of prehybridizing solution (P1415, Sigma) which contained 1 N Danhardt’s salt, formamide (50% final concentration), salmon sperm DNA (500 μg/ml) and yeast transfer RNA (250 μg/ml). The sections were hybridized at 56°C overnight in 50 μl hybridizing cocktail which contained the same ingredients as the prehybridization solution with the addition of 50 ng of either sense control or antisense probes and dextran sulphate (10% final concentration). Post-hybridization stringency washes included three brief rinses with 2×SSC, one rinse in RNase buffer (10 mM Tris, 1 mM EDTA and 500 mM NaCl, pH 8.0), and digestion with RNase A (50 μg/ml) and RNase T1 (2 units/ml) for 1 h at 37°C in RNase buffer. Post-RNase digestion washes were carried out in a shaking water bath and included RNase buffer for 30 min at 37°C, 0.1×SSC for 15 min at 50°C and 0.1×SSC for 15 min at 37°C.

**Detection of digoxigenin activity**

After stringency washing, the sections were rinsed briefly with Tris/NaCl buffer (100 mM Tris, 150 mM NaCl, pH 7.5). Non-specific binding of the antibody was blocked by incubating the sections with 2% normal sheep serum (Boehringer Mannheim) and 0.1% Triton X100 in Tris/NaCl buffer at room temperature for 30 min. The sections were incubated with 100–200 μl of an anti-digoxigenin polyclonal antibody conjugated to alkaline phosphatase (Boehringer Mannheim), at 1/5000 dilution in Tris/NaCl buffer, for 2 h at room temperature. The sections were washed briefly in Tris/NaCl buffer and then with Tris/NaCl/MgCl2 buffer (100 mM Tris, 100 mM NaCl, 50 mM MgCl2, pH 9.5). Endogenous phosphatase activity was blocked by incubating the sections with 5 mM levamisole (Sigma) in Tris/NaCl/MgCl2 buffer for 20 min at room temperature. The alkaline phosphatase activity was detected by incubating the sections overnight at room temperature with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium (NBT) according to Boehringer Mannheim’s instruction, i.e. 1.8 mg/ml X-phosphate and 3.375 mg/ml NBT, in Tris/NaCl/MgCl2 buffer. The sections were washed in RO water and mounted with an aqueous mount (Clearmount™, Zymed Laboratories, San Francisco, CA, USA). The specificity of the signal was validated in an independent run using sections of a progesterone receptor-positive breast tumour tissue and a proliferative endometrial tissue. It was found that prehybridizing the sections with ×10 (i.e. 500 ng) non-labelled antisense probe for 2 h significantly reduced the signal intensity, whereas similar treatment with the non-labelled sense probe had no effect on signal intensity. Omission of the antisense probe (i.e., mock run) did not produce a signal. In addition, pretreatment of the sections with RNase A (20 μg/ml, 37°C, 30 min) blocked the signal whereas pretreatment with RNase-free RNase I (400 IU/ml) increased the signal slightly. Sections of the progesterone receptor-positive breast tumour tissue and proliferative endometrial tissue were then included in every run as positive controls.

**Data analyses**

The expression of progesterone receptor mRNA in the tissues was assessed by semi-quantitative scoring of the staining intensity, where no staining = 0, little staining = 1, moderate staining = 2 and intense staining = 3. The scoring was carried out blind and all samples were scored in one session to reduce variability. The data were analysed by one-way analysis of variance. To evaluate the effect that length of post-fixation storage in PBS had on progesterone receptor mRNA detection, a linear regression analysis was performed.
Progesterone receptor mRNA in normal and Norplant endometrium

Results

Regression analysis showed no difference in the progesterone receptor mRNA signal intensity regardless of the length of time of sample storage in PBS. The regression coefficients ($r^2$) for the glands and stroma were 0.044 ($P = 0.25$) and 0.062 ($P = 0.14$) respectively.

Progesterone receptor mRNA was detected in the glandular epithelium and stromal cells at all stages of the menstrual cycle (Figure 1). The concentrations of progesterone receptor mRNA staining in the glandular epithelium varied during the menstrual cycle. During the early proliferative stage of the menstrual cycle, there was little progesterone receptor mRNA staining in the glands with some glands almost devoid of staining. The concentrations of progesterone receptor mRNA staining in the glands increased thereafter and there was significantly more staining during stage 2 than stage 1 ($P < 0.01$; Figure 2). There was a trend for lower concentrations of progesterone receptor mRNA staining during stage 3 than stage 2 although the difference was not statistically significant. In contrast, the concentrations of progesterone receptor mRNA staining in the stroma was consistent and no variation was found across the menstrual cycle.

Progesterone receptor mRNA was detected in glandular epithelia and stromal cells of the majority of Norplant endometria (Figure 1). When compared with the normal menstrual cycle, Norplant endometrium had significantly less progesterone receptor mRNA staining in glandular epithelia than stage 2 of the cycle ($P < 0.01$; Figure 2). A similar reduction was
also observed in the Norplant stroma compared with stroma in normal endometrium during stages 2 (\(P < 0.05\); Figure 2) and 3 (\(P < 0.01\); Figure 2).

The Norplant amenorrhoea group (i.e. no bleeding/spotting days in the 90 days prior to biopsy) had significantly (\(P < 0.05\)) more progesterone receptor mRNA staining in the stroma than the Norplant bleeding group (bleeding ranging from 4 to 78 days in the 90 days prior to biopsy), as shown in Figure 3. A similar increase was also observed in the glandular epithelium but the difference was not statistically significant.

The plasma concentrations of oestrogen were significantly (\(P < 0.05\)) lower in the amenorrhoea Norplant group (200.8 ± 74.0 pmol/l) than the bleeding Norplant group (523.9 ± 77.0 pmol/l). In contrast, no difference in the plasma progesterone concentrations between the two groups was observed (1.30 ± 0.71 and 1.29 ± 0.32 nmol/l respectively).

### Discussion

In the present study using in-situ hybridization, we have found that glandular concentrations of progesterone receptor mRNA vary significantly during the menstrual cycle, while stromal concentrations remain unchanged. Glandular progesterone receptor mRNA concentrations were significantly higher in the latter part of the proliferative phase compared to earlier in the menstrual cycle. Values fell from this peak during the secretory phase, although this reduction was not statistically significant (the present study). However, there appears to be a differential sensitivity of different endometrial tissue compartments to steroid regulation of progesterone receptor expression. Despite glandular epithelium and the stromal cells being to the same hormonal milieu, progesterone receptor proteins (Critchley et al., 1993) and their mRNA (the present study) vary only in the glandular epithelium but remain relatively steady in stromal cells throughout the menstrual cycle. The mechanism controlling this differential sensitivity is not understood. One possibility is that the stromal progesterone receptor and its mRNA are more stable than the glandular progesterone receptor and its mRNA. Alternatively, there may be differential expression of the A and B forms of progesterone receptors (Kastner et al., 1990) in different tissue compartments of the endometrium and different forms of progesterone receptor may respond differently to the same steroid influence.

We have previously found that Norplant endometrial stroma had significantly higher concentrations of immunoreactive progesterone receptor than that of the normal menstrual cycle, while no such difference was observed for the glandular epithelium between the two groups (Critchley et al., 1993). In contrast, the present study found that progesterone receptor mRNA concentrations were lower in Norplant than that in the normal endometrium during the latter part of the proliferative phase (for both stroma and glands) and secretory phase (for stroma). The underlying reason for these apparent discrepancies is not known. The rate of turnover of progesterone receptor and its mRNA in the human endometrium is not known. It is possible that levonorgestrel may have a negative effect on both the transcription of progesterone receptor and the mechanism controlling the degradation of progesterone receptors thus leading to a reduction in mRNA concentrations and a concomitant stabilization of the progesterone receptor proteins. It is also possible that the increase in stromal progesterone receptor

**Figure 3.** Progesterone receptor mRNA staining intensity score in endometrial glandular epithelium (solid bars) and stromal cells (open bars) of Norplant subjects with amenorrhea or spotting/bleeding (ranging from 4 to 78 days over the 90 days prior to biopsy). \(*P < 0.05\) compared with the same tissue compartment.
Progestosterone receptor mRNA in normal and Norplant endometrium

protein detected by immunohistochemistry was a result of the detection of the metabolic products of the receptor proteins. Although the endometrial samples from the normal menstrual cycle and Norplant endometrial samples were of different racial origins, these discrepancies are unlikely to be due to racial differences since the same sets of samples from the normal menstrual cycle and Norplant users were used in the present and previous studies on progesterone receptor proteins (Crichtley et al., 1993). In addition, Norplant induces the same degrees of breakthrough bleeding in Caucasian women (Shoupe et al., 1991; Frank et al., 1993) as in Asian women (Singh et al., 1989; Akhter et al., 1993).

The reduction in endometrial stromal progesterone receptor mRNA concentrations in Norplant users compared with those in the normal menstrual cycle is unlikely to be due to the lack of oestrogenic stimulation. In fact, most Norplant users have plasma oestrogen concentrations indicative of follicular activity (Brache et al., 1985; present study). In addition, plasma oestrogen concentrations were lower but progesterone receptor mRNA concentrations were higher in the amenorrhoea than the bleeding groups (the present study) while no difference in oestrogen receptor protein concentrations between the amenorrhoea and bleeding groups was found (Crichtley et al., 1993).

Collectively, these data indicate that oestrogen may not be the only stimulatory factor in the transcription of progesterone receptors in the human endometrium. This suggestion is consistent with the observation of differential regulation of progesterone receptor protein and mRNA in stroma versus glands during the menstrual cycle, despite both tissue compartments being subject to similar oestrogen exposure. Further studies are required before a fuller understanding of the physiological implication of this observation can be made.

The result showing higher progesterone receptor mRNA concentrations coupled with lower plasma oestrogen concentrations in the Norplant amenorrhoea group compared with the bleeding group is a significant finding. The association between reduced endogenous oestrogen concentrations and reduced breakthrough bleeding in users of long-term progestin-only contraceptives has been reported before (Hadisaputra et al., 1996) and inferred from the study of White et al. (1991), where increased breakthrough bleeding was correlated with epoxide production, which in turn was correlated with circulating oestrogen concentrations. These reports, coupled with the results of the current study, appear to confirm a significant relationship between reduced endogenous oestrogen concentrations and reduced breakthrough bleeding in users of progestin-only contraceptives. Consequently to this finding, a paradox emerges as to why treatment with ethinyl oestradiol appears to be successful in reducing prolonged and/or irregular bleeding in women receiving similar contraceptive regimes (Diaz et al., 1990; Witjaksono et al., 1996). Further studies on the relationship between breakthrough bleeding and oestrogen are clearly required.

The positive relationship between increased endometrial progesterone receptor mRNA expression and amenorrhoea found in the present study is one of the few demonstrations of an association between an endometrial parameter and bleeding patterns. One previous study reported an association between endometrial epoxide production and increased bleeding (White et al., 1991). Apart from this, numerous studies of a range of variables, including endometrial steroid receptors (Crichtley et al., 1993), steroid hormone-binding globulin (Brache et al., 1992), endometrial microvasculature density (Rogers et al., 1993), endometrial endothelial cell proliferation (Goodger et al., 1994) and endothelin production (Marsh et al., 1995) have failed to show any relationship with bleeding.

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