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

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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).

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, Neullin-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 were categorized into three main stages of the normal menstrual cycle (Rogers et al., 1993) as follows: menstrual to early proliferative (namely stage 1, i.e. at a time of low plasma oestrogen concentration,
The sections were fixed in 4% paraformaldehyde in KPBS (263 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 4 mM NaCl, 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 KPBS (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 Denhardt’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 prehybridizing 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 2X 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, 0.1X SSC for 15 min at 50°C and 0.1X 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 DNase 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.

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Progesterone receptor mRNA in normal and Norplant endometrium

Figure 1. Endometrial progesterone receptor mRNA staining pattern during the normal menstrual cycle (a–f) and in Norplant users (g–i) (a) Early proliferative, antisense; (b) mid-proliferative, antisense; (c) late proliferative, antisense; (d) early secretory antisense; (e) late secretory, antisense; (f) late proliferative, sense control; (g) Norplant subject with amenorrhoea; (h) Norplant subject with 22 spotting/bleeding days; and (i) Norplant subject with 56 spotting/bleeding days over the 90 days prior to endometrial biopsy.

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.

Figure 2. Progesterone receptor mRNA staining intensity score in endometrial glandular epithelium (solid bars) and stromal cells (open bars) of controls during the different stages of the menstrual cycle and of Norplant users. Mean ± SEM M-EP (i.e stage 1) = menstrual to early proliferative, EMP-LP (i.e stage 2) = early-to-mid proliferative to late proliferative, and SECR (i.e stage 3) = secretory. *$P < 0.05$ (stage 1 vs stage 2) **$P < 0.01$ (Norplant vs controls of the same tissue compartments)

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 epithelium than stage 2 of the cycle ($P < 0.01$; Figure 2) A similar reduction was
et al. (1996) are most likely due to the different
of Ingamells
The differences in results between the present study and that
proteins and its mRNA concentrations (Ingamells
and that there was no correlation between progesterone receptor
progesterone receptor mRNA across the normal menstrual cycle
was no variation in the concentrations of endometrial pro-
receptor mRNA are also reduced during the secretory phase, plasma progesterone concentrations
are elevated and the expression of glandular progesterone
receptor proteins are reduced (Press et al., 1988; Critchley et al., 1993). The concentrations of glandular progesterone
receptor mRNA are also reduced during the secretory phase
although the reduction is not statistically significant (the present
study). However, there appears to be a differential sensitivity
of different endometrial tissue compartments to steroid regula-
tion of progesterone receptor expression. Despite glandular epithelia 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 epithelia but remain relatively steady in stromal cells through-
out 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 differ-
ently 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

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. These patterns of progesterone receptor mRNA
expression are consistent with that observed previously for
progesterone receptor proteins in both the glands and stroma
(Press et al., 1988; Critchley et al., 1993; Ingamells et al.,
1996).

A recent study, using Northern analyses, showed that there
was no variation in the concentrations of endometrial pro-
gesterone receptor mRNA across the normal menstrual cycle
and that there was no correlation between progesterone receptor
proteins and its mRNA concentrations (Ingamells et al., 1996).
The differences in results between the present study and that
of Ingamells et al. (1996) are most likely due to the different

![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.](https://academic.oup.com/humrep/article-abstract/11/12/2629/71452/149205)
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 (Critchley et al., 1993). In addition, Norplant induces the same degree 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 that 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 (Critchley et al., 1993). Collectively, these data indicate that progesterone mRNA concentrations 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 results 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 (Hadasaputra 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 (Critchley et al., 1993), steroid hormone-binding globulin (Brache et al., 1992), endometrial microvasculature density (Rogers et al., 1993), endometrial endothelial cell proliferation (Goodyer et al., 1994) and endothelin production (Marsh et al., 1995) have failed to show any relationship with bleeding.

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