Endometrial progesterone and estrogen receptors and bleeding disturbances in depot medroxyprogesterone acetate users

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BACKGROUND: Depot medroxyprogesterone acetate (DMPA) is a very popular hormonal contraceptive. Unpredictable bleeding disturbances are the main reasons for discontinuation and may be mediated through endometrial hormone receptors. This study was aimed to compare the expression of progesterone and estrogen receptors in the endometrium of bleeding DMPA users with that of amenorrhoeic DMPA users. METHODS: Subjects were recruited between April 2000 and January 2001. On the day of the third DMPA injection, 42 amenorrhoeic DMPA users and 42 DMPA users who had frequent or prolonged endometrial bleeding and were bleeding on that day were matched by age and body mass index. Endometrial biopsies were collected for immunohistochemical study of progesterone receptor A plus B (PRAB) and B alone (PRB) and estrogen receptor α (ERα) and β (ERβ) expression. RESULTS: There were 23 adequate endometrial samples from each group. There were no differences in any of a series of comparisons of PRAB, PRB, ERα and ERβ expression in glands or stroma between the groups. Serum estradiol and progesterone levels were also not different between the groups. CONCLUSIONS: Endometrial PRAB, PRB, ERα and ERβ expression in glands and stroma was not different between DMPA users who had frequent or prolonged bleeding and amenorrhoeic DMPA users.

Key words: depot medroxyprogesterone acetate/endometrial bleeding/estrogen receptor/progesterone receptor/progestogen

Introduction

Progestogen-only contraceptives are among the most effective and increasingly popular methods for contraception. A total of ~20×10⁶ women worldwide currently use these methods. Depot medroxyprogesterone acetate (DMPA) is the original injectable progestogen-only contraceptive and is currently used by 13×10⁶ women (d’Arcangues, 2000). Injection of 150 mg of DMPA every 84–90 days provides extremely high contraceptive efficacy. Failure rates range from 0 to 0.7 per 100 woman-years, which is comparable with surgical sterilization (Kauzit, 2001). However, bleeding disturbances from DMPA and the other progestogen-only contraceptives are the main reasons for discontinuation. The discontinuation rate of DMPA due to bleeding disturbances was generally 21–28 per 100 women at 1 year of use, accounting for 52–68% of all reasons for discontinuation (Fraser, 1999). A number of treatments are used by clinicians but none of them appears sufficiently effective because the mechanisms of bleeding disturbances related to progestogen-only contraceptives are still not well understood (d’Arcangues, 2000; Hickey and Fraser, 2000).

Cyclical variations of circulating estradiol and progesterone levels provide the stimulus for normal endometrial development and menstruation. Aberrations in the cyclical variation of these hormones can cause spontaneous abnormalities of endometrial bleeding. Estradiol and progesterone act on endometrium primarily through their own receptors. Therefore, abnormal expression of these receptors may also be involved in the mechanisms of disturbances of endometrial bleeding.

The human PR is expressed as two isoforms, progesterone receptor A (PRA) and progesterone receptor B (PRB). PRA differs from PRB by lacking 164 amino acids from the N-terminus (Kastner et al., 1990). During the menstrual cycle, PRA and PRB are expressed at comparable levels in the epithelial compartment but PRA is predominant in the stromal compartment (Mote et al., 1999). Both PRA and PRB function...
as ligand-activated transcription factors, but it has been suggested on the basis of in vitro studies that the two isoforms are not functionally equivalent. Transient co-transfection of PRA or PRB and progestogen-sensitive reporter genes has shown that PRA is transcriptionally more active than PRA (Wen et al., 1994; Giangrande et al., 1997). Furthermore, PRA can act as a dominant repressor of PRB activation of progestogen-sensitive reporter genes (Wen et al., 1994; Mote et al., 1999). Therefore, the relative levels of PRA and PRB within the endometrium may determine the functional responses to progestogens.

Estrogen receptors also occur in two isoforms, estrogen receptor α (ERα) and β (ERβ). The classical estrogen receptor, ERα, has been known and well characterized for many years. ERβ was first cloned from a rat prostate complementary DNA library in 1996 (Kuiper et al., 1996). Homologues have now been identified in many species, including human (Mosselman et al., 1996; Enmark et al., 1997). ERα and ERβ share common structural features exhibiting variable degrees of homology, with the highest level of homology present in the DNA-binding (C) and ligand-binding (E) domains (Cavaillès, 2002). Studies in vitro have demonstrated that although both ERα and ERβ bind estradiol with equal affinity (Kuiper et al., 1997), these receptors may have differential responses to some estrogen agonists and antagonists (Watanabe et al., 1997; Barkhem et al., 1998; Sun et al., 1999). Studies in ERα-knockout and ERβ-knockout mice have shown that ERβ may modulate the effects of ERα on the endometrial progesterone receptor expression in response to estradiol (Kurita et al., 2000; Weihua et al., 2000). Studies in mammary tissues of the rat have suggested that one role of ERβ may be to antagonize ERα-mediated actions in epithelial cells (Saji et al., 2000), a function supported by data from in vitro cell transfections (Hall and McDonnell, 1999) but not yet confirmed in vivo. Therefore, the proportion of ERα and ERβ expression in the endometrium could determine the response to estradiol and its analogues.

Previous studies about PR and progestogen-only contraceptives have yielded conflicting results. In subdermal levonorgestrel implant (Norplant) studies, PR immunostaining in the endometrial stromal compartment was higher than controls (Crichtley et al., 1993), but progesterone receptor mRNA concentrations were lower than controls (Lau et al., 1996). PRB immunostaining of glandular and stromal compartments was much lower than PRA in Norplant users (Glasier et al., 2002). In levonorgestrel-releasing intrauterine system studies, PR was down-regulated in both endometrial glands and stroma (Janne and Ylostalo, 1980; Salmi et al., 1998; Zhu et al., 1999) with lower PRB than PRA (Crichtley et al., 1998). In the DMPA study, the endometrium at the bleeding sites had lower PRA and PRB expression than the endometrium at the non-bleeding sites (Lockwood et al., 2000). However, there has been no study of PRA and PRB expression in the endometrium of amenorrhoeic DMPA users compared to bleeding DMPA users.

In the Norplant and levonorgestrel-releasing intrauterine system studies, ERα expression was lower than in control proliferative endometrium but there was no difference in the expression of ERβ with respect to the bleeding pattern (Crichtley et al., 1993, 1998; Zhu et al., 1999). However, they did not study ERβ. There has also been no study of ERα and ERβ expression in endometrium of DMPA users.

Therefore, the aim of the present study was to compare the endometrial progesterone receptor A and B (PRAB), PRB, ERα and ERβ in DMPA users who experienced bleeding disturbances with endometrial PRAB, PRB, ERα and ERβ from DMPA users who experienced amenorrhoea.

Materials and methods

Subjects

This study used endometrial samples from a previously published clinical study (Chotnopparatpattara et al., 2003). The clinical details are as follows. Eighty-four healthy, fertile, non-lactating women, aged between 25 and 35 years, were recruited from the family planning clinic at King Chulalongkorn Memorial hospital between April 2000 and January 2001. All subjects had no known medical or gynaecological disorders, nor previous history of chronic anovulation. They also had no previous history of abortion, or endometrial curettage. Subjects reporting any other hormonal treatment were excluded. All subjects had one child and had received 150 mg of DMPA as contraception at 6 weeks postpartum, with continued injection approximately every 84 days. Menstrual data were recorded on a menstrual diary chart. Ethical approval for the study was obtained from the Ethics Committee of the Chulalongkorn University. On the day of the third DMPA injection, subjects were divided into two groups according to the occurrence of bleeding in the previous reference period; an amenorrhoeic group and a bleeding group. Both groups had used DMPA for >168 days. The reference period was defined as 84 days after the last DMPA injection. Amenorrhoea was defined as no genital bleeding in the reference period. The presence of bleeding was defined as a significant amount of genital bleeding such that the use of sanitary pads was anticipated in the reference period. Women who had frequent or prolonged bleeding, defined as bleeding episodes which were more frequent than four times or lasted >10 days in one reference period (Belsey and Pinol, 1997) and were biopsied during bleeding, were included in the bleeding group. All participants who had missed >10 days of menstrual data collection in the reference period were excluded.

Forty-two subjects in each group were matched for age and body mass index. All subjects signed written informed consent. Before DMPA injection, blood was collected. Serum was separated, stored at −20°C and subsequently analyzed for estradiol (Delfia, Finland) and progesterone (Delfia) by time-resolved fluoroimmunoassay. The inter-assay and intra-assay variations for estradiol were 3.9 and 3.7% respectively. The inter-assay and intra-assay variations for progesterone were 3.9 and 3.7% respectively. The inter-assay and intra-assay variations for progesterone were 3.9 and 3.7% respectively.

Endometrial specimens were obtained by Endocell biopsy (Wallach Surgical Devices, Inc., USA). All endometrial tissue samples were fixed overnight in 10% buffered formalin and paraffin-embedded using a routine method. Sections 5 μm thick were stained with haematoxylin and eosin for histological assessment and also were collected on 3-aminopropyltriethoxysilane (APTS)-coated slides for immunohistochemical study.
Histological assessment
Histological assessment of the endometrial biopsies was performed by an experienced gynaecological pathologist (P.R.) who was blinded to the patient characteristics. The biopsies were classified as normal proliferative phase, normal secretory phase, inactive or resting, suppressed secretory, early secretory with underdeveloped glands and irregular secretory endometria (Maqueo et al., 1970).

Immunohistochemistry
Immunohistochemical studies were done at the Department of Obstetrics and Gynaecology, Faculty of Medicine, University of Sydney. The sections on 3-APTS-coated slides were deparaffinized and rehydrated to distilled water. Antigen retrieval was carried out by heating the sections in the Target Antigen Retrieval Solution® (Dako Corp., USA) in a microwave oven set on high power for 15 min. After cooling for 20 min, slides were washed in 0.05 mol/l Tris-buffered saline (TBS) and endogenous peroxidase was blocked with 3% hydrogen peroxide. Non-specific binding of the primary antibody was blocked by incubating the slides with 5% swine serum for 5 min. Since specific antibody for PRA for use with formalin-fixed tissue could not be obtained for this study, an antibody detecting both PRA and PRB was utilized (PRAB). Primary antibodies for PRAB (diluted 1:100, NCL-PGR-AB; Novocastra Laboratories, UK) and PRB (diluted 1:100, NCL-PGR-B; Novocastra Laboratories) were applied on the slides and incubated at room temperature for 60 min. Primary antibody for ERα (diluted 1:200, M7047, Clone 1D5; Dako Corp.) and ERβ (diluted 1:200, 385P; BioGenex, USA) were applied on the slides and incubated at room temperature for 30 min. Biotinylated antimouse/rabbit antibody and streptavidin conjugated to horseradish peroxidase (Dako LSAB®+, Kit; Dako Corp.) were then added sequentially and incubated for 15 min each. Staining was visualized by addition of substrate and chromogen 3,3’-diaminobenzidine (Dako® Liquid DAB+; Dako Corp.) for 5 min. All tissue sections were counterstained with Mayer’s haematoxylin, dehydrated and cleared in xylol then mounted with UltraMount.

Proliferative endometrium sections were used as positive controls. For negative controls, the sections were incubated with IgG from same animal species which produced primary antibodies (mouse or rabbit IgG) at a matched concentration in place of primary antibody.

Assessment of immunostaining
All tissue sections were blindly assessed by the same observer (W.S.). Counting and modified H-score techniques were validated by comparison of blinded assessments between different experienced observers. Endometrial glands and stroma were assessed separately. Quantitative analysis of positive PRAB, PRB, ERα and ERβ immunostaining was performed by counting the positive stained cells. Ten random fields from each section were counted with a grid-eye piece pre-calibrated with a slide micrometer at a high power magnification (×400). The number of positive cells was expressed per 1000 epithelial or stromal cells.

Semi-quantitative analysis of cell number and staining intensity was also performed using modified H-scores (Ravn et al., 1993). First, the fraction (F) of stained cells in each compartment was estimated: 0 = 0–9%, 1 = 10–39%, 2 = 40–69%, 3 = 70–89% and 4 = 90–100%. Second, the staining intensity (I) was scored: 0 = no staining, 1 = weak but definite staining, 2 = moderate staining, 3 = pronounced staining and 4 = intense staining. Finally, the H-score was calculated by the formula: Σ(F×I)/4.

Statistical analysis
Data were analysed using the Statistical Package for Social Science (SPSS) software for Windows 10.0 (SPSS Inc., USA). Intra-observer and inter-observer comparisons were assessed by intra-class correlations. Comparisons between groups were made using the paired t-tests for normally distributed data and the Wilcoxon signed rank tests for abnormally distributed data. Correlations between variables were analysed using the Spearman’s rank correlation coefficients. Two-sided P < 0.05 was considered statistically significant.

Results
Forty-two women in each group were recruited. Twenty-seven women from the amenorrhoeic group and 30 women from the bleeding group had adequate endometrial tissue for analysis. Due to the match-paired design of our study, we excluded four women from the amenorrhoeic group and seven women from bleeding group who did not match with women in another group. Therefore, we had 23 women from each group for analysis. Table I summarizes the clinical characteristics in each group. Age, weight, height, body mass index, serum estradiol and progesterone were not different between groups.

Blinded histological assessment reported the following endometrial appearances: three normal proliferative phase, seven inactive or resting, nine suppressed secretory and four early secretory endometria with underdeveloped glands in the amenorrhoeic group. In the bleeding group, there were one normal proliferative phase, six inactive or resting, 11 suppressed secretory and five early secretory endometria with underdeveloped glands. There were no statistical differences in histological findings between groups.

The intra-observer and inter-observer correlation coefficients for the counting technique were 0.951 (P < 0.001) and 0.906 (P < 0.001) respectively. The intra-observer and inter-observer correlation coefficients for the modified H-score technique were 0.926 (P < 0.01) and 0.902 (P < 0.01) respectively.

Table II summarizes the endometrial PRAB, PRB, ERα and ERβ expression in each group. The numbers of positive cells per 1000 total cells and modified H-scores of the endometrial PRAB, PRB, ERα and ERβ in glands and stroma were not different between groups.

The mean number of bleeding days per reference period in the bleeding group was 34.1 ± 24.5 days (mean ± SD), ranging from 5 to 84 days. The number of bleeding days did not correlate with the endometrial PRAB, PRB, ERα and ERβ expression. Serum estradiol correlated positively with the

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<tr>
<th>Table I. Clinical characteristics of amenorrhoeic and bleeding groups of women using depot medroxyprogesterone acetate</th>
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<tr>
<td>Clinical characteristics</td>
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a Mean ± SD.  
b Paired t-test.  
c Mean ± SEM.  
d Wilcoxon signed rank test.
modified H-score of glandular PRB \( (r = 0.358, P = 0.017) \). Serum estradiol inversely correlated with the number of glandular cells positively expressing ER\(\alpha\) per 1000 total cells \( (r = -0.374, P = 0.012) \). Serum estradiol did not correlate with the glandular PRAB, stromal PRAB, stromal PRB, stromal ER\(\alpha\), glandular ER\(\beta\) nor stromal ER\(\beta\) expression. Serum progesterone did not correlate with PRAB, PRB, ER\(\alpha\) nor ER\(\beta\) expression in either glands or stroma.

### Discussion

This study has demonstrated that endometrial PRAB, PRB, ER\(\alpha\) and ER\(\beta\) expression in DMPA users who had frequent or prolonged bleeding were not different from DMPA users who had amenorrhoea. A good primary antibody for detecting PRA and PRB with equal efficiency (PRAB). A specific antibody for PRB was available. We have assumed that PRA is the subtype responsible for immunostaining when positive PRAB staining was detected but PRB staining was not detected (Critchley et al., 1998; Glasier et al., 2002). Therefore, from our results, we have concluded that the endometrial PRA expression in DMPA users who had frequent or prolonged bleeding was not different from DMPA users who had amenorrhoea.

We used the number of positive cells per 1000 total cells to measure the PRAB, PRB, ER\(\alpha\) and ER\(\beta\) immunostaining results because their staining located sharply in the nucleus, allowing accurate counting. However, an assessment of the number of positive cells does not take account of the number of receptors in each cell. The modified H-score is a semi-quantitative method developed to include a measure of the intensity of immunostaining, as well as the proportion of positive cells. The modified H-score should therefore provide a relative assessment of the total amount of receptors in the tissue. We have validated the assessment of immunostaining by high levels of intra-observer and inter-observer correlation of counting and modified H-score techniques.

This study design was match-paired, in order to eliminate major confounding factors between groups. The women in both groups had similar clinical characteristics apart from their bleeding experience with DMPA. They were matched primarily for age and body mass index. All women were started on DMPA at 6 weeks postpartum and had used DMPA for ~168 days. Biopsies in the bleeding group were obtained only during bleeding episodes to clearly distinguish between amenorrhoeic and bleeding groups.

We obtained 46 endometrial biopsy specimens from 84 women that provided adequate tissue for analysis. From previous studies, 15% of endometrial samples from DMPA users who received two doses of DMPA were found to be inactive (Maqueo et al., 1970). It can be very difficult to obtain endometrial tissue from these women by aspiration biopsy. We used an Endocell biopsy device because it causes less pain to the women than a conventional curette, and it can be performed in an outpatient setting. We have assumed that inadequate endometrial tissue in the biopsies in our study was most likely due to atrophic endometrial changes from the DMPA. Previous biopsy studies in progestogen-only contraceptive users have also found inadequate tissue collection from endometrial biopsy in similar proportions of subjects (Hadisaputra et al., 1996; Zhu et al., 1999). However, these should have little effect on data analysis in this study because the numbers of women who had inadequate endometrial samples in each group were quite similar and our match-paired study design should have taken this into account.

Most of the studies on the progestogen-only contraceptives have demonstrated reduced endometrial progesterone receptor
content compared to endometrium from normally cycling women (Janne and Ylostalo, 1980; Lau et al., 1996; Critchley et al., 1998; Zhu et al., 1999). Only one study demonstrated that the endometrial PR concentrations in Norplant users were higher than normal women (Critchley et al., 1993). Our study did not compare with normal women, therefore we could not evaluate these findings. Based on studies of endometrium in normal menstrual cycles, progesterone in the secretory phase down-regulates expression of its own receptor (Graham and Clarke, 1997). Chronic and prolonged exposure to the progestogen-only contraceptives may have a similar effect to progesterone in secretory phase endometrium as demonstrated by other studies (Janne and Ylostalo, 1980; Lau et al., 1996; Critchley et al., 1998; Zhu et al., 1999).

Our results have clearly demonstrated that endometrial PRAB and PRB expression (which presumably also applies to PRA) was not different in amenorrhoeic DMPA users and DMPA users who had frequent or prolonged bleeding. These results are different from the Norplant study which showed lower progesterone receptor mRNA expression in the bleeding group (Lau et al., 1996). This disagreement may be due to different type or concentration of progestogens between DMPA and Norplant. Lockwood et al. (2000) have studied in DMPA users and demonstrated that the endometrium at bleeding sites in DMPA users had lower PRA and PRB expression than the endometrium at non-bleeding sites within each user. However, they did not compare amenorrhoeic users with frequent or prolonged bleeding users. There were only six subjects in their study and they did not clarify bleeding pattern in their subjects. They identified apparent bleeding sites by hysteroscopy and the relevance of this to frank, clinical endometrial bleeding is not yet clear. Our results also showed that the number of bleeding days in the bleeding group was not correlated with endometrial PRA and PRB expression. Therefore, based on our results, the endometrial PRA and PRB expression, in either glands or stroma, does not appear to be directly linked to the phenomenon of endometrial breakthrough bleeding in DMPA users.

In the Norplant and levonorgestrel-releasing intrauterine system studies, the endometrial estrogen receptors α (ERα) were lower than the control proliferative endometrium (Critchley et al., 1993, 1998; Zhu et al., 1999). These findings result from the known inhibiting effect of progestogen on estrogen receptor expression (Graham and Clarke, 1997). Our study did not include normal cycling women, therefore we could not confirm this finding. Our study has demonstrated that ERα and ERβ expression was not different between the amenorrhoeic and bleeding groups in both glandular and stromal compartments. These findings agree with the previous study in Norplant users which showed that ERα expression did not correlate with the number of bleeding days (Critchley et al., 1993). Our results have also shown that serum estradiol levels were not different between the amenorrhoeic and bleeding groups. Therefore, it appears likely that the actions of estrogen through its endometrial receptors, both ERα and ERβ, do not differ between DMPA users who had frequent or prolonged bleeding and DMPA users who had amenorrhoea.

This study has shown that serum estradiol and progesterone levels were not different between amenorrhoeic and bleeding groups. Our results are again different from the Norplant study, which showed lower serum estradiol in the amenorrhoeic group (Lau et al., 1996). This could be due to different type or concentration of progestogens between DMPA and Norplant. Serum estradiol in DMPA users, as confirmed in this study, is much lower than in Norplant users (Lau et al., 1996). Therefore, serum estradiol in DMPA users should have less influence on the endometrium than in Norplant users.

In this study, glandular PRB expression correlated positively with serum estradiol concentrations. Glandular ERα expression inversely correlated with serum estradiol. In normal menstrual cycles, high circulating levels of estradiol in the proliferative phase induces both PRA and PRB synthesis, and progesterone in the secretory phase down-regulates expression of both PRA and PRB (Graham and Clarke, 1997; Mote et al., 2000; Critchley et al., 2001). This difference between DMPA users and normal women may be the result of differences in the hormonal milieu. The clinical significance of the correlation between endometrial PRB and ERα expression and serum estradiol in DMPA users is unclear. This correlation does not extend to differences in the amenorrhoeic and bleeding groups.

Belsy et al. have reported that bleeding patterns in DMPA users varied with ethnic groups (Belsey and Peregoudov, 1988). In this study, we recruited only Thai DMPA users. There may be some limitations of generalizing our results to other ethnic groups.

Many studies on progestogen-only contraceptives have shown potential mechanisms that might relate to endometrial bleeding. These include changes in endometrial vascular morphology, changes in vascular structural integrity, increased capacity for cell breakdown, changes in endometrial perfusion and oxygenation, changes in endometrial responsiveness to sex steroids, changes in endometrial vascular haemostasis and changes in the immunocompetent cells in endometrium (Fraser, 1999; Hickey and Fraser, 2000; Smith, 2000). Further studies are still needed to clarify some of these mechanisms and to define effective treatments.

In conclusion, endometrial PRAB, PRB, ERα and ERβ expression in both glands and stroma in amenorrhoeic DMPA users was not different from DMPA users who had frequent or prolonged bleeding. Therefore, there may be no direct influence of progesterone and estrogen receptor expression on episodes of active endometrial bleeding, although there could still be subtle influences at a post-receptor level. It is more likely that the main mechanisms involve factors unrelated to progesterone and estrogen receptor expression.

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References


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