A prospective randomized controlled study comparing the morphological and biochemical responses of the endometrium to two different forms of ‘period-free’ hormone replacement therapy

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Thirty postmenopausal women were randomized to receive either continuous combined (cc) 2 mg oestradiol valerate and 0.7 mg norethisterone acetate hormone replacement therapy (HRT) daily (15 women) or tibolone 2.5 mg daily (15 women) and were monitored to determine the relationship between the two biochemical markers placental protein 14 (PP14) and the glycoprotein CA125, endometrial histology and occurrence of irregular bleeding after 12 months of treatment. The concentrations of PP14 and CA125 in plasma and uterine flushings before and after therapy were measured and their concentrations were associated with the histology of endometrial biopsies obtained on the same day as venesection and endometrial flushing. The levels of PP14 in uterine flushings were significantly increased after the administration of both types of HRT (P < 0.05 for tibolone and P < 0.001 for ccHRT). However, the concentrations of PP14 found in flushings after ccHRT were considerably greater than those found in flushings after tibolone; levels were increased about 150-fold by ccHRT and 6-fold by tibolone (P < 0.001). Plasma concentration of PP14 after both types of HRT were also significantly raised to a similar degree (P < 0.01). In contrast, the concentration of plasma and uterine CA125 were unchanged by either treatment. Histological analysis of the endometrium from women after 12 months of HRT treatment showed that 86% (6/7) of women with higher post-HRT uterine PP14 concentration were more likely to have irregular bleeding (P < 0.05). Our studies have shown that endometrial PP14 but not CA125 concentrations are raised to a significant degree by two different forms of period-free HRT regimens. Increased PP14 concentrations in uterine flushing may suggest endometrial stimulation of some form and predict the predilection to irregular bleeding. Thus uterine PP14 concentrations may be used to monitor endometrial responses in women on HRT.

Key words: CA125/continuous combined oestrogen–progesteron therapy/placental protein 14/postmenopausal/tibolone

Introduction

The multiple benefits of hormone replacement therapy (HRT) are well documented (Gambrell, 1982; MacLennan, 1991), yet only between 10 and 16% of women who qualify for HRT receive a prescription and of these only ~30% continue to take the prescription after one year (National Opinion Poll, 1991). This is usually for a shorter period than that required to derive the maximum benefits (e.g. prophylaxis against osteoporosis and cardiovascular morbidity and mortality requires administration for at least 5 years). Some of the reasons advanced for the low uptake and poor compliance include extreme progesterin intolerance (Panay and Studd, 1997) and withdrawal and/or irregular bleeding (Okon et al., 1996). Period-free HRT was developed in an attempt to obviate withdrawal bleeding and therefore improve uptake and compliance with HRT. Irrespective of the HRT regimen used, some women still develop irregular bleeding and monitoring of the endometrium, particularly in long term users and in those who develop irregular bleeding, may be necessary to exclude the development of endometrial neoplasia.

Traditionally, the study of endometrial function has been based on histological examination of endometrial biopsy specimens. The introduction of refined histological techniques such as morphometry (Li et al., 1988), histochemistry (Klentzeris et al., 1991) immunohistochemistry (Bell et al., 1985) and computer-assisted three-dimensional evaluation (Casanas-Roux et al., 1996) has permitted a more detailed study of the various components of the endometrium. More recently, endometrial physiology has been studied by the analysis of uterine protein content (Li et al., 1993a) and the patterns of distribution of these proteins (Beier-Hellwig et al., 1989) in endometrial secretions obtained by direct aspiration or by the technique of uterine flushing (Li et al., 1993b).

One of the endometrial proteins which reflects the secretory activity of the endometrium in premenopausal women is placental protein 14 (PP14). This glycoprotein, secreted by the endometrium, starts to rise in the luteal phase and reaches its highest levels in the plasma and uterine fluids in the luteal phase (Joshi et al., 1986; Julkunen et al., 1986; Li et al., 1993a). Another glycoprotein produced by the endometrium is CA125 (Jacobs and Bast, 1989). Levels of CA125 in uterine flushings have been shown to correlate with uterine PP14 (Dalton et al., 1995). It has previously been shown that plasma PP14 levels go up in women receiving HRT (Li et al., 1992) but to date, few data are available on the concentrations of these two proteins in uterine flushing from women on HRT.

The object of this study was to compare the morphology and biochemical response of the endometrium to two different forms of period-free HRT, and to determine the relationship between the biochemical markers PP14 and CA125, endometrial histology, and bleeding pattern.
Materials and methods

Subjects
Thirty postmenopausal women [defined as those women who have at least 12 months of amenorrhoea and with plasma follicle stimulating hormone (FSH) of $>20$ IU/l but not older than 65 years] with an intact uterus who requested period-free hormone replacement therapy were recruited for this study. The women were randomized into two groups and given either continuous combined HRT using Climesse® (micronized oestradiol valerate 2 mg and norethisterone 0.7 mg; Sandoz Pharmaceuticals, Camberley, Surrey, UK; 15 women) or Livial® (tibolone 2.5 mg; Organon Pharmaceuticals, Cambridge, UK; 15 women) daily for 12 months. None of the women had taken any steroid hormones for 3 months prior to entering the study. The study was approved by the local ethics committee and informed consent was obtained from each woman. Prior to randomization, all the subjects had blood sampling for baseline plasma FSH, CA125 and PP14, uterine flushing and biopsy. They were requested to record bleeding if any on a daily basis. At the end of this 12 month period, blood sampling, uterine flushing and endometrial biopsy were repeated in the last week of the calendar pack.

Uterine flushing
Flushing was done using a modification of the technique previously published (Li et al., 1993b). This was performed as an outpatient procedure using the Pipelle® endometrial sampler (Laboratoire CCD, Paris, France) instead of a Foley catheter. A bivalve speculum was inserted into the vagina and through it a Pipelle® endometrial sampler, preloaded with 1 ml of sterile normal physiological saline (0.9% NaCl), was slowly introduced into the uterus. After ~30 s, the tip of the sampler was withdrawn by ~2 cm into the lower part of the uterine cavity, the saline solution was then aspirated slowly through the vacuum mechanism of the Pipelle® sampler. The plunger was withdrawn by ~2 cm at a time, altogether taking ~30 s. A volume of ~0.8 ml was consistently aspirated. The aspirate was immediately centrifuged at 2200 g for 5 min, to remove any tissue contaminants and the supernatant was stored at ~20°C for PP14 and CA125 assay.

Endometrial biopsy
Immediately after the uterine flushing, an endometrial biopsy specimen was obtained by use of the same Pipelle® sampler. Each biopsy was fixed immediately in buffered glutaraldehyde and sent to the pathology laboratory for processing and analysis. The fixed tissue was embedded in glycomethacrylate (JB4) and stained with acid fuchsin and Toluidine Blue before being sectioned for examination by light microscopy. The endometrial biopsies were examined histologically with the use of the dating criteria of Noyes et al. (1950).

PP14 assay
PP14 was measured by radioimmunoassay using the method previously described by Bolton et al. (1987). In brief, PP14 was iodinated by the chloramine-T method and the resulting tracer was purified using a column of conA Sepharose. For the assay, 100 µl of 1 ng/ml PP14 tracer and 100 µl of standards or sample were incubated at room temperature for 24 h with 100 µl antiserum, at a dilution to bind ~45% of the added tracer. The antibody bound tracer was separated from the unbound using Amerlex-MMT magnetic separating reagent (Amersham International, Little Chalfont, Bucks, UK). The sensitivity of the assay was 3 ng/ml, and the intra- and inter-assay coefficients of variation were <10%.

CA125 assay
CA125 concentrations in serum and uterine flushings were measured at the Immunology Department of the Northern General Hospital, Sheffield, using an ELSA-CA125 kit (CIS Bio International, Cedex, France), a two site immunoradiometric assay. The CA125 was adsorbed onto the solid phase by antibody ELSA-CA125 11 and then quantified using $^{125}$I monoclonal antibody as tracer. All assays were carried out in duplicate and performed according to the instruction of the kit manufacturers. The lower limit of detection was 7 IU/l and the intra- and inter-assay coefficients of variation were <5%.

Follicle stimulating hormone assay
FSH was measured by immunoenzymetric assay (magnetic solid phase) using the human FSH Serozyme Kit (Serono Laboratories, Welwyn Garden City, Herts, UK). The sensitivity of the assay was 0.3 mIU/ml and the intra- and inter-assay coefficient of variation were <6%.

Analysis of data
The concentrations of PP14, CA125 and FSH from the tibolone and continuous combined HRT groups were compared using Student’s $t$-test.

Results
The mean ($\pm$SD) ages of women on ccHRT and tibolone groups were 55.0 ($\pm$2.8) and 54.8 ($\pm$3) years respectively. The mean interval from menopause to entry into the study was 57.5 ($\pm$32) and 67.5 ($\pm$33) months respectively. There was no significant difference in mean age and interval from the menopause between the two groups. For personal reasons, 10 women withdrew from the study. One of the women on ccHRT was subsequently found to have an ovarian cyst and was therefore excluded from the analysis. Nine of the 15 women on ccHRT and 10 out of 15 on tibolone completed the study.

The mean ($\pm$SEM) concentrations of PP14 in plasma before and following treatment with ccHRT were 20.2 ($\pm$11) and 69.7 (34.9) ng/ml respectively and 11.1 ($\pm$4.4) and 22.7 ($\pm$1.7) ng/ml for the tibolone group. The mean uterine flushing PP14 concentrations before and after 12 months on ccHRT were 8 ($\pm$4.3) and 1416 ($\pm$229) ng/ml respectively, and 10.1 ($\pm$5.5) and 73 ($\pm$25) ng/ml for the tibolone-treated women. Low concentrations of PP14 were found in both the plasma and uterine flushings obtained from women before HRT. The concentrations of PP14 in the uterine flushings were significantly increased by both types of HRT ($P < 0.05$ for tibolone and $P < 0.001$ for ccHRT). However, the concentration of PP14 in the flushings after ccHRT were considerably greater than those found in flushings after tibolone; concentrations were increased ~150-fold by ccHRT and 6-fold by tibolone ($P < 0.001$). PP14 in the plasma after both types of HRT were also significantly higher ($P < 0.01$) compared to those found before treatment. Nonetheless, the plasma PP14 was only increased by ~5-fold and there was no significant difference in the plasma PP14 between the women on tibolone and ccHRT.

The mean concentration of PP14 in uterine flushings after 1 year of treatment in women who bled during the treatment (1105 ng/ml) was significantly higher than those who did not bleed (269 ng/ml) (Figure 1). There was no significant difference in the mean number of days of bleeding (9, 95% CI 2–16) in women taking ccHRT as compared to tibolone (6, 95% CI 2–10).
The mean (±SEM) concentrations of CA125 in plasma before and following treatment were 19 (±4) and 16 (±2) for the ccHRT group and 16 (±2) and 17 (±3) for the tibolone group. Similarly, the mean (±SEM) levels in the uterine fluid before and after treatment were 25 254 (±11 386) and 13 022 (±3024) for the ccHRT group and 24 301 (±7067) and 18 660 (±3015) respectively. Unlike PP14, the CA125 levels in either plasma or uterine flushings were not significantly changed by either form of HRT. There was a considerable variation in the levels of CA125 found, particularly in the flushings.

Figure 2 shows the effects of ccHRT and tibolone on plasma FSH. Both types of treatment caused a significant reduction in plasma FSH concentration with ccHRT producing a 6-fold reduction and tibolone a 2-fold reduction.

For one woman on ccHRT and one on tibolone, there was not enough tissue for analysis. In the ccHRT group 86% (6/7) of endometrial specimens showed secretory activity as compared to 44% (4/9) from women on tibolone. The remaining specimens showed atrophic changes. Figure 3 shows the relationship between histological classification of the endometrial tissue from women on both types of HRT plotted against the concentrations of PP14 found in the uterine flushings obtained on the same day. There was no significant difference in the amount of uterine PP14 in women with atrophic endometrium compared with those who had secretory endometrium.

Discussion

In this study we have shown that both types of period-free HRT produced a significant rise in the concentration of PP14 in plasma and uterine flushings. The increase in PP14 levels was more marked in women treated with ccHRT compared to those receiving tibolone confirming that there is a significant difference in endometrial secretory activity or response in the two regimens of period-free HRT. These findings are consistent with our current understanding that tibolone is a gonadomimetic steroid with weak oestrogenic properties which is expected to produce minimal endometrial stimulation. Our study also showed that ccHRT produced a significantly greater degree of suppression of plasma FSH concentration than tibolone.

We were unable to show a significant correlation between the histological assessment of endometrial development and the concentration of PP14 in uterine flushings, perhaps owing to the relatively small number of subjects in the study. Therefore, larger numbers may be required to determine statistically the relationship. Alternatively, it may suggest a limitation of the use of histology in assessing endometrial secretory activity, which is in keeping with previous studies that suggested no correlation between endometrial biological activity, as reflected by histology, and cytosol progesterone receptor profiles (Bayard et al., 1978). Nevertheless, the presence of atrophy on histology may well represent extreme progestin effect and would be clinically reassuring. On the other hand, it may also mean insufficient circulating oestrogen, as oestrogen priming of the endometrium is required for progestin action. However, a low oestrogen level was unlikely, as the 2 mg micronized oestradiol used in this study has been shown to produce endometrial proliferation (Casanas-Roux et al., 1996). This same study reported secretory transformation in all the endometrial specimens following progesterone treatment. These findings could be explained, firstly, by the short duration of treatment, and secondly, by the cyclical sequential
method of administration as compared to ours which was continuous combined. This difference would affect the induction of oestrogen and progesterone receptors in the endometrium which determines luteal transformation. In general, however, with endometrial atrophy the PP14 concentration ought to be low, as observed at the onset of the study. It may be that the progestogenic effect includes increased leakage of the PP14 from the cells due to changes in membrane integrity. It is of interest that there was an association between breakthrough bleeding and high uterine PP14 concentrations which may reflect changes in the vascular endothelium (Casanas-Roux et al., 1996).

In contrast to PP14, there was no significant change in the concentration of CA125 in the serum and flushings following HRT treatment. This result is somewhat surprising as we have previously shown that in premenopausal women CA125 and PP14 levels in flushings correlate well (Dalton et al., 1995). This suggests that the production of CA125 in the postmenopausal woman may not be directly dependent on sex steroids. The relatively high CA125 found in the flushings of postmenopausal women prior to HRT treatment was unexpected considering the atrophic nature of the endometrium in these women. It is known that CA125 is produced in other organs such as the ovaries, but it is uncertain if the CA125 was from these sources. There is a paucity of data on endometrial flushing and plasma CA125 in postmenopausal women with endometriosis. McBean and Brumsted (1993), reported a 2- to 4-fold increase in the production of CA125 by endometrial cells in culture from premenopausal women with confirmed endometriosis. We cannot exclude the possibility of endometriosis in our study population, but as the women were postmenopausal and asymptomatic before entry into the study, endometriosis if present would probably have been quiescent.

It has been reported that cytosolic CA125 concentrations in the endometrium were 20-fold and 2-fold higher than those measured in the Fallopian tube of premenopausal women respectively (Zeimet et al., 1993). Moreover, only in the endometrium did CA125 content show significant cyclic changes, with the highest concentrations during the early proliferative and middle secretory phase.

Our results suggest that PP14 is a better biochemical marker than CA125 in monitoring of HRT in postmenopausal women and that levels >270 ng/ml in uterine flushing are more probably associated with breakthrough bleeding. Although the numbers in the study are low, striking differences in the concentrations of uterine PP14 are seen in the women on cCrHRT and tibolone. This was not obvious when the response was assessed by histological examination of the endometrial biopsy specimens. Another alternative to monitoring the endometrial response in women on HRT is transvaginal ultrasound scan (Osners et al., 1990; Castelo-Branco et al., 1994; Wolman et al., 1996). An ultrasound scan thickness of <4 mm is associated with a very low risk of endometrial pathology.

In conclusion our studies have shown that endometrial PP14 concentrations are raised to significantly different degrees by two different forms of HRT regimens. Women with higher uterine PP14 following a period of HRT treatment also had a higher predilection to irregular bleeding. Due to the small size of our series, the conclusions are drawn with caution. Further larger studies are required to clarify this and to determine whether similar differences are seen in other HRT regimens, but the results suggest that the measurement of uterine PP14 levels may be a useful tool to assess the endometrial response in women on HRT, particularly when minimal changes are being monitored, and in predicting those likely to bleed whilst on period-free HRT. If the changes are found to correlate with endometrial hyperplasia and/or malignancy then it may become an adjunct to, or replace, other methods for screening.

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