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ORIGINAL ARTICLE

Percutaneous estradiol/oral micronized progesterone has less-adverse effects and different gene regulations than oral conjugated equine estrogens/medroxyprogesterone acetate in the breasts of healthy women *in vivo*

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Gene expression analysis of healthy postmenopausal women in a prospective clinical study indicated that genes encoding for epithelial proliferation markers Ki-67 and progesterone receptor B mRNA are differentially expressed in women using hormone therapy (HT) with natural versus synthetic estrogens. Two 28-day cycles of daily estradiol (E2) gel 1.5 mg and oral micronized progesterone (P) 200 mg/day for the last 14 days of each cycle did not significantly increase breast epithelial proliferation (Ki-67 MIB-1 positive cells) at the cell level nor at the mRNA level (MKI-67 gene). A borderline significant beneficial reduction in anti-apoptotic protein bcl-2, favouring apoptosis, was also seen followed by a slight numeric decrease of its mRNA. By contrast, two 28-day cycles of daily oral conjugated equine estrogens (CEE) 0.625 mg and oral medroxyprogesterone acetate (MPA) 5 mg for the last 14 days of each cycle significantly increased proliferation at both the cell level and at the mRNA level, and significantly enhanced mammographic breast density, an important risk factor for breast cancer. In addition, CEE/MPA affected around 2,500 genes compared with just 600 affected by E2/P. These results suggest that HT with natural estrogens affects a much smaller number of genes and has less-adverse effects on the normal breast *in vivo* than conventional, synthetic therapy.

Keywords: bcl-2 protein, gene expression, HT, micronized progesterone, normal breast tissue, percutaneous estradiol, proliferation

Introduction

Hormone therapy (HT) has been associated with an increased risk for breast cancer in postmenopausal women. Various preparations, doses and regimens of HT can have different effects; combined estrogen–progestogen therapy confers a greater increase in breast cancer risk than estrogen monotherapy [1–6], and there is evidence to suggest that topical rather than systemic use of HT may lessen the increase in risk [7]. Although the effects of added progestogens are not uniform [8–15], breast cell proliferation response is typically seen within 2 months of initiating combination therapy [10,11,15]. The use of synthetic versus natural progestogens appears to increase this response. Several studies have shown increased breast cell proliferation in response to sequential conjugated equine estrogens–medroxyprogesterone acetate (CEE–MPA) [12,13,16–19], whereas treatment with percutaneous estradiol and oral natural micronized progesterone (E2-P) is not significantly associated with this potentially hazardous side effect [19,20].

In the breast, cell proliferation marker Ki-67 is an important biological marker for the risk of developing malignancy. However, the clinical significance of changes in this marker is incompletely understood. We have previously shown in healthy postmenopausal women a trend-level significant increase ($p = 0.05$) in the number of Ki-67–positive cells with CEE–MPA versus E2-P and a significant downregulation of bcl-2 protein ($p = 0.01$ for total material of both treatments), favouring apoptosis [21]. In the context of increased breast cell proliferation and the associated increase in risk of transformation to the neoplastic phenotype, a reasonably higher rate of apoptosis (as indicated by a reduction in the anti-apoptotic protein bcl-2) should be considered beneficial [21]. In this study, we report on the observed adverse effects and gene regulations in the breasts of healthy women *in vivo*.

Subjects and methods

A prospective clinical study was performed in apparently healthy postmenopausal women aged 44–66 years at the Karolinska University Hospital, Stockholm, Sweden, between May 2006 and 2008. Eligible women were non-smokers who had been postmenopausal for at least 12 months with normal mammogram results, a body mass index of 18 to 30 kg/m^2, follicle-stimulating hormone levels >25 IU/L and E2 levels <90 pmol/L. A minimum washout period for previous HT users of 3 months was imposed. Exclusion criteria were any heart disease, previous breast surgery, hepatic dysfunction, active gallbladder disease, or history of thromboembolic disease. Medication with sexual steroids, barbiturates, carbamazepines, phenytoin, glucocorticoids, rifampicin, cimetidine, diltiazem, erythromycin, ketoconazole, verapamil, and quinidine was not permitted.

The study was approved by the independent ethics committee IRB-2005/762-31 and the Swedish Medical Products Agency EU-2005/001016-51. All women gave their written informed consent.

The women were randomized to sequential HT with two 28-day cycles of daily oral 0.625 mg CEE or 2.5 g 0.06% percutaneous E2 gel (1.5 mg E2), with the respective addition

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of oral 5 mg MPA or oral 200 mg natural P for the last 14 days of each cycle. Quantification of circulating sex steroid and sex hormone-binding globulin levels by routine hospital methods was done before treatment and on one of the last 3 days of the second 28-day treatment cycle (days 54–56). Concentrations of free testosterone were calculated as described by Södergård and colleagues [22].

Mammographic study

Women with high mammographic density are at increased risk for breast cancer, and several studies have shown that a number of estrogen-progestogen–related risk factors for breast cancer, such as HT, are also associated with mammographic density [23]. Mammography was performed at baseline to assess the density of the breasts. Mammograms were analyzed by a radiologist with expertise in mammography and classified according to the Breast Imaging – Reporting and Data System (BI-RADS) visual analogue scale. Dense areas were correlated to the total area of the mammogram in four steps: <25%, 26–50%, 51–75% and >75% dense areas. A second mammogram was performed during one of the last 3 days of the second 28-day treatment cycle (days 54–56).

Biopsy study

Percutaneous stereotactic core needle biopsy (2.0 mm) was performed at baseline in the area of highest mammographic density of the upper lateral quadrant of the left breast with the patient under local anaesthesia on a prone table (Lorad, DSM, Danbury, CT). A repeat biopsy in the same area was performed at days 54–56, always on the same day as circulating sex steroid and sex hormone-binding globulin levels were measured. Two specimens from each biopsy were analyzed by immunohistochemistry as described in [21].

Gene expression study

A third specimen from each biopsy was taken for gene expression analysis by microarray (Affymetrix, Santa Clara, CA, USA) and real-time PCR (RT-PCR). Consistent with previous microarray studies of estrogen-mediated genes [24–27], we used a fold change cut-off point of ±1.5 to represent an important change in gene expression. Detection of Ki-67 was performed using the MIB-1 monoclonal antibody (Bench Mark, Ventana Medical Systems, Illkirch Cedex, France), which reacts with a human nuclear antigen present in proliferating cells and absent in quiescent cells [28]. Detection of bcl-2 protein was performed using the commercially available antibody bcl-2 clone 124 (nr 760–4246; Cell Marque Corp., Rocklin, CA, USA) [29]. We also looked at progesterone receptor B (PgRB) gene expression as an additional marker of breast cell sensitivity for progestogens. Estrogen mediates the relative expression of PgRB and PgRA in the breast by inducing PgR mRNA and protein in breast cells [30].

Statistical methods

Differences between the two treatment groups were assessed with use of the Mann–Whitney test. For within-group changes the Wilcoxon signed-rank test was used for immunohistochemistry data and the Student's *t*-test for microarray data. Correlations were assessed by Spearman's rank correlation test. A p value <0.05 was considered statistically significant.

Results

In total, 99 women were tested for eligibility. Twenty-two women were excluded for not meeting the inclusion criteria. Seventy-seven women were assigned randomly to treatment. A total of 71 women completed the study. Six women (1 receiving conjugated equine estrogens-MPA and 5 receiving E2-P) discontinued treatment.

Mammographic study

Preliminary results from the mammographic study showed a significant increase in mammographic density of at least one BI-RADS density class in seven of 37 patients (18.9%) using synthetic hormones (CEE/MPA group) between baseline and days 54–56 ($p = 0.01$). By contrast, only two of the 32 patients (6.3%) using natural hormones (E2/P group) had an increase in mammographic density of at least one density class over the same period (n.s.).

Biopsy study

Of the 71 women who completed the study, 35 (49%) had assessable breast epithelium at both biopsy time points. In these women, after 2 months of treatment, CEE/MPA but not E2/P induced a significant increase in breast cell proliferation ($p = 0.003$ and n.s., respectively), as described in [21], with a between-group difference of borderline significance ($p = 0.05$) (Table I), illustrated in Figure 1. The percentage of bcl-2–positive cells at days 54–56 was not reported in one woman using E2/P due to a slightly different cellularity between different sections of the same biopsy.

Gene expression study

A total of around 33,000 genes were analyzed by microarray. In women using synthetic estrogen (CEE/MPA), the expression of approximately 2,500 genes in breast cells was altered with absolute fold change ≥1.5, compared with around 600 genes in women using natural estrogens (E2/P). Approximately 300 genes with altered expression by fold change ≥1.5, were common to both treatment groups.

Gene expression data are currently available in eight women with sufficient amount of extractable mRNA at both biopsy time points: four in each treatment group. In these women, E2/P did not induce a significant fold change from baseline biopsy in MKi-67, bcl-2 or PgRB gene expression to biopsy at days 54–56. The largest numerical differential expression in women using E2/P was a fold-change 1.4 increase in PgRB mRNA (Table II). By comparison, in the CEE/MPA group, significant differential expression of MKi-67 and PgRB was seen with positive fold

Table I. Results from biopsies at baseline and at days 54–56 of treatment.

	Ki-67 MIB-1–positive cells			Bcl-2–positive cells		
	Baseline	Days 54–56	*p* value	Baseline	Days 54–56	*p* value
E2/P ($n = 17$)*						
Mean	3.1%	5.8%	n.s.	50.6%	26.5%	0.06
Median	1.4%	1.8%		50.0	22.5%	
CEE/MPA ($n = 18$)						
Mean	1.0%	10.0%	0.003	42.3%	26.3%	n.s.
Median	0.6%	2.5%		50.0%	15.0%	
Treatment difference	–	–	0.05	–	–	n.s.

*Bcl-2 data not available in one patient at baseline or at days 54–56.
Data expressed as % of positive cells. n.s. = nonsignificant. No significant difference between treatment groups at baseline for either assay.

Table II. Gene fold change (days 54–56 vs. baseline).

	MKi-67 gene fold change ($n = 4$)	Bcl-2 gene fold change ($n = 4$)	PGR (B) gene fold change ($n = 4$)
E2/P	+1.12 (n.s.)	−1.35 (n.s.)	+1.4 (n.s.)
CEE/MPA	+1.93 ($p = 0.014$)	−1.31 (n.s.)	+2.6 ($p = 0.01$)

n.s., nonsignificant.

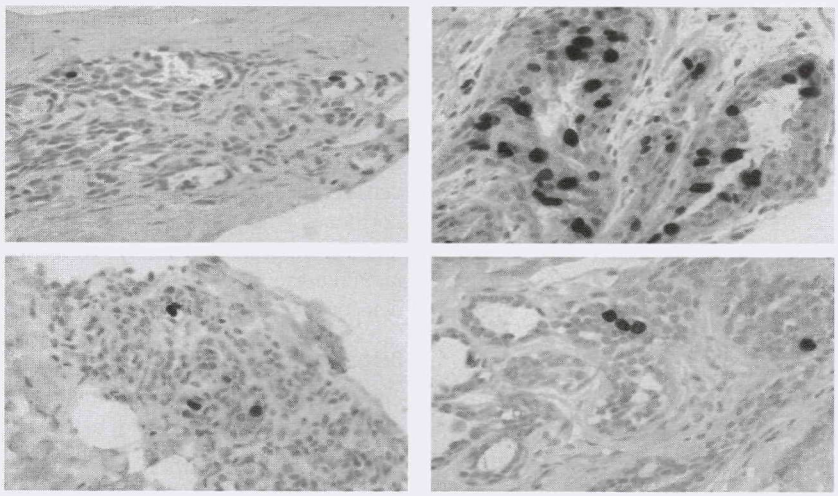

Figure 1. Breast histology from two individual women before (left) and after 2 months (right) of sequential treatment with either oral CEE/MPA (above) or percutaneous E2/oral P (below). Nuclei of proliferating cells stained brown by Ki-67 MIB-1 antibody. Reprinted with permission from Murkes et al. [21]. [Subject to permission from Fertil Steril].

changes of 1.93 and 2.6 ($p = 0.014$ and $p = 0.01$, respectively). Fold change in bcl-2 gene expression was similar in both groups (E2/P-1.35, CEE/MPA-1.31). Early results from the RT-PCR analysis are fairly consistent with the microarray data (data not shown). Further patients are being analyzed.

Discussion

Core needle biopsy has made repeated extraction of high-quality mRNA from healthy postmenopausal women *in vivo* possible. Generally, the larger amount of material retrieved during biopsy allows for assessment of protein content as well as gene expression analysis by both microarray and RT-PCR, thus allowing patients to act as their own controls. In our study, however, the proportion of women with assessable breast epithelium in biopsies both before and after 2 months of treatment was unexpectedly low (49% versus the assumed yield of ≥55% used in power calculations) [21]. Consistent with previous studies in breast tissue [31,32], none of the women experienced complications of core needle biopsy.

In this study, a markedly higher number of genes were affected by CEE/MPA than by E2/P. Preliminary data from Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA, USA) of the microarray results indicate major differences in several canonical signalling pathways between treatment with CEE/MPA vs. E2/P (data not shown). Our group has previously demonstrated a significant positive correlation between breast epithelial proliferation (Ki-67 positivity) and insulin-like growth factor 1 (IGF-1) mRNA during hormonal treatment in eight premenopausal women using synthetic hormonal contraception which was not found in women with untreated menstrual cycles [33]. This suggests that the proliferative response of breast tissue to HT goes beyond the direct action of sex steroid receptors to potentially include a role for IGF-1 [33], a known mitogen for several cancer cell lines [34,35], and we continue to explore this possibility by using IPA and RT- PCR-analysis.

Another potentially important mediator of the effects of combined HT was recently explored by Courtin and colleagues, who found that glucocorticoid receptor activity discriminated between the mitogenic effects of E2+MPA and E2+P treatment, particularly in cell proliferation and apoptosis gene families [36]. These opposing effects was observed in both normal luminal breast cells and in cancerous breast cells, suggesting that MPA could increase tumorigenesis by promoting proliferation in the former and cancer progression in the latter according to progesterone and glucocorticoid receptor status [36]. However, one important caveat when considering all three of these studies in the current context is that data are available for only a small number of healthy postmenopausal women, thereby limiting the robustness of any statistical inference from comparisons between individual treatment groups.

In addition to canonical pathway considerations of the apparent differential effects on gene activation of synthetic and natural HT, the route of estrogen administration may be one explanation for the large discrepancy between the number of genes affected by CEE/MPA and E2/P. In the large General Practitioners Database, systemic treatment with oral estrogens but not transdermal topical estrogens significantly increased breast cancer risk [7,37,38]. A study in perimenopausal women by Schmidt and colleagues had previously shown that topical treatment with 0.01% E2 significantly improved the appearance of skin in the applied area without increasing systemic levels of estrogen [39].

The ultimate goal of combined HT, especially in the breast, is to define treatment regimens and alternatives for postmenopausal women that provide effective relief from climacteric symptoms with minimal adverse effects on healthy epithelium. The findings of this study suggest that the effects on normal breast proliferation *in vivo* and mammographic density of two 28-day cycles of treatment with percutaneous E2 gel, plus natural micronized P for the last 14 days of each cycle, are less adverse than the effects of the conventional synthetic alternative. The markedly different gene regulations seen in women using CEE/MPA versus E2/P may offer further insight into this differential effect on breast epithelium, and should be explored in further studies.

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