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Report

Effects of oral contraceptives on breast epithelial proliferation

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Summary

The association between oral contraceptive (OC) use and breast cancer is not fully understood. Estrogen is a known mitogen to breast epithelial cells, but there is still a controversy about the effect of added progestogens. Fine needle aspiration (FNA) biopsies were used to assess epithelial proliferation in normal breast tissue from 106 healthy premenopausal women with and without oral contraceptives. In 26 women biopsies were performed before and after 2 months of OC use. Proliferation, expressed as percentage of Ki-67/MIB-1 positive cells, was correlated to endogenous progesterone, androgenic/anabolic compounds and exogenous progestogen. We found a higher proliferation ($p = 0.03$) in OC users compared to non users, with mean values of 4.8% and 2.2%, respectively. There was a positive correlation between proliferation and progesterone levels in non-users and with serum levonorgestrel concentrations in women using OCs containing this progestogen ($rs = 0.43$, $p = 0.02$). Women using OCs had significantly lower serum androgen levels compared to naturally cycling women and free testosterone levels displayed an inverse relation to breast epithelial proliferation. There was a marked variation in the response to exogenous sex steroids. In certain women after 2 months of OC use, the percentage of MIB-1 positive cells was as high as 40–50%. The results add to the growing evidence that progestogens may be mitogenic in breast tissue. Increased proliferation during hormonal contraception should be regarded as an unwanted and potentially hazardous side effect. Efforts should be made to define hormonal contraceptive regimens which minimize breast epithelial proliferation and to identify those women with the most pronounced proliferative response.

Introduction

Different combinations of estrogen and progestogen are used for contraception and hormonal replacement therapy by numerous women worldwide. The possibility of an increased risk of breast cancer in relation to such treatment is vividly discussed. Since breast cancer is the most common malignancy in women in the western world and the use of hormonal treatment is so abundant, it is clear that even a small increase in risk is of major importance for women's health. While our understanding of the association between oral contraceptives (OC) and breast cancer is incomplete, a pooled reanalysis of 54 epidemiological studies demonstrated an increased risk in current users. After cessation of use, the excess risk was normalized within 5–10 years [1].

The basis of an increased risk associated with hormonal therapies may lie in the regulation of cell proliferation. Within populations of cells *in vitro* and *in vivo* high rates of cell proliferation increase the risk of transformation to the neoplastic phenotype [2]. *In vitro* studies have previously suggested a regulation of the breast epithelium similar to that of the endometrium where enhanced proliferation is seen during estrogen stimulation and inhibited proliferation is found after treatment with progestogens [3, 4]. However, *in vivo* studies of the breast have shown increased proliferation during the luteal phase of the menstrual cycle when both estrogen and progesterone levels are high

[5, 6]. Previous studies on the effects of OC use on breast tissue have been mainly analyses of mitoses in tissue sections from reduction mammoplasties or from 'normal' breast tissue near benign or malignant lesions [7, 8]. Studies that are based on such tissue specimens have apparent limitations and proliferation analysis is possible at only one occasion.

Fine-needle aspiration (FNA) biopsy is an established technique for the pre-operative diagnosis of palpable lumps in the breast [9]. Numerous reports have shown a high correlation and reproducibility between the cytology from FNAs and histological follow-up from open biopsies or surgical specimens [10, 11]. Estrogen and progesterone receptors can be measured in aspirated cells through immunocytochemistry [12, 13]. Monoclonal antibodies against cell proliferation specific antigens are also available for the assessment of proliferation in cytologic breast cell samples [14]. The cytospin technique yields samples with a higher cellularity than conventional smears and allows evaluation of proliferation in FNA biopsies [6]. We have previously shown that it is possible to evaluate proliferation and sex steroid hormone receptors status through FNA biopsies from the breasts of young, healthy women during the normal menstrual cycle [6, 13].

In this study, FNA biopsies were used to assess epithelial cell proliferation in normal breast tissue from healthy women who were users and non-users of oral contraceptives. A subgroup of women was also investigated before and after 2 months of OC use. Breast epithelial proliferation was correlated to endogenous progesterone and androgenic/anabolic compounds and to exogenous progestogen, that is levonorgestrel.

Materials and methods

Subjects

A total of 106 healthy premenopausal women without any history or symptoms of breast disease were recruited for FNA. There were 41 OC users, mean age 26.1 years (18–50). The OCs contained ethinyl estradiol and different progestogens, that is levonorgestrel ($n = 11$), desogestrel ($n = 18$), lynestrenol ($n = 3$), norethisterone ($n = 9$). In women using OCs the FNA was performed during day 16–21 of treatment. For comparison, 39 women, mean age 31.6 years (21–45), who did not use OCs were analyzed by FNA during the second half of the menstrual cycle. In addition, in 26 women FNA was repeatedly performed before and after 2 months of OC (ethinyl estradiol/levonorgestrel) use, mean age in this group was 28.8 years (19–45). All women had regular menstrual cycles. Venous blood samples were drawn and anamnestic menstrual data were collected at the day of FNA. The women who did not use OCs had not taken any sex steroid containing drugs during the last 6 months preceeding the study. The study was approved by the Local Ethics Committee and all women gave their informed consent.

Fine needle aspiration biopsy

Percutanous FNA biopsy of the upper outer quadrant of the left breast was performed after palpation to determine the area with the highest density with a needle of 0.6 mm as described by Franzén and Zajicek [9]. To produce several identical slides the aspirated cells were mixed with 0.5 ml of 4% buffered (pH 7.4) formalin in the same syringe as the procured cells. Volumes of 110 µl were cytocentrifuged at 700 rpm for 3 min and enriched epithelial cells were spotted on pretreated glass slides.

Assessment of proliferation

Immune-stained cells were quantified through cell counting by an observer blinded to treatments given. The Ki-67/MIB-1 monoclonal antibody reacts with a human nuclear antigen which is present in proliferating, but absent in quiescent cells. Cell cycle analysis shows that the antigen is expressed in the phases of G1, S, G2 and mitosis [14]. The MIB-1 analyses were performed with reagents supplied by Immunotech, Marseilles, France. The staining procedure uses an avidin–biotin peroxidase system, modified for the cytospin technique. On average aspirates yielded 400–600 cells per slide, and in all cases a minimum of 50 epithelial cells were scored. We considered samples obtained by FNA to be assessable only if they contained intact epithelial cells and not free-lying nuclei.

Analytical methods

Serum concentrations of progesterone and sex hormone-binding globulin (SHBG) were determined by chemiluminiscence immunoassay using a commercial kit (Immulite®) obtained from Diagnostic Products Corporation (DPC), Los Angeles, CA.

Testosterone (T) was determined by radioimmunoassay in untreated serum using a commercial kit (Coat-a-Count® Testosterone) obtained from DPC. 4-androstene-3,17-dione (A-4) in serum was determined after extraction with diethyl ether by radioimmunoassay as previously described [15, 16]. Serum levels of insulin-like growth factor I (IGF-I) were determined by radioimmunoassay after acid ethanol extraction with a commercial kit from Nichols Products Corporation, San Juan Capistrano, CA. Detection limits and within and between assay coefficients of variation were for progesterone 0.6 nmol/l, 6% and 7%; for SHBG 0.05 nmol/l, 4% and 8%; for T 0.1 nmol/l, 6% and 10%; for A-4 0.6 nmol/l, 6% and 10% and for IGF-I 0.6 μg/l, 6% and 10%, respectively.

Apparent concentrations of free testosterone (fT) were calculated from values for total T, SHBG and a fixed albumin concentration of 40 g/l by successive approximation using a computer program based upon an equation system derived from the law of mass action [17].

Plasma levels of levonorgestrel were determined by radioimmunoassay after extraction with diethyl ether according to Weiner and Johansson [18] with slight modifications according to Olsson [19]. Anti-levonorgestrel 11α-hemisuccinate-bovine serum albumin (rabbit) and tracer (15,16 [^{3}H]-d-norgestrel, specific activity 30–50 Ci per mmol) were obtained from Schering AG, Berlin, Germany. Because of a small plasma blank, the practical detection limit was 25 pg per tube. No corrections were made for procedural losses, nor were the plasma blanks subtracted. The extraction recovery was 89–95%. With the extraction volume of 200 μl, the detection limit was 0.16 nmol/l. For random samples the within assay coefficient of variation was 9% for samples <1.0 nmol/l and 6% for those >1.0 nmol/l and the between assay coefficient of variation was 11% for samples <1.0 nmol/l and 8% for those >1.0 nmol/l. The antibody did not cross react with any naturally occurring steroids.

Statistical analysis

Differences between groups were analyzed by the Mann–Whitney U test or one way ANOVA. The Wilcoxon signed rank test was used for paired observations. Data were tested for normal distribution by the Kolmogorov–Smirnov test. Normally distributed data were expressed as arithmetic mean ±S.E.M., otherwise as median and range. Correlations were assessed by Spearman's rank and Pearson's regression analysis. A p-value of <0.05 was considered statistically significant.

Results

From the 106 women a total number of 132 FNA biopsies were obtained and 107 (81%) of these were evaluable for MIB-1 content. The 25 remaining biopsies were non-evaluable due to too few cells in the aspirates.

Breast epithelial proliferation as expressed by the percentage of MIB-1 positive cells was significantly higher ($p = 0.03$) in women using OCs as compared to non-users (Figure 1). Values were mean 2.2%, median 1.5% and range 0–8% in women not using OCs and 4.8%, 3.0% and 0–50% in the group of OC users. Regression analysis did not reveal any significant influence of age on breast epithelial proliferation neither in the whole study population nor in the OC users and non-users. No differences between women younger and older than 35 years were found by ANOVA. Parity had no apparent influence on the expression of MIB-1

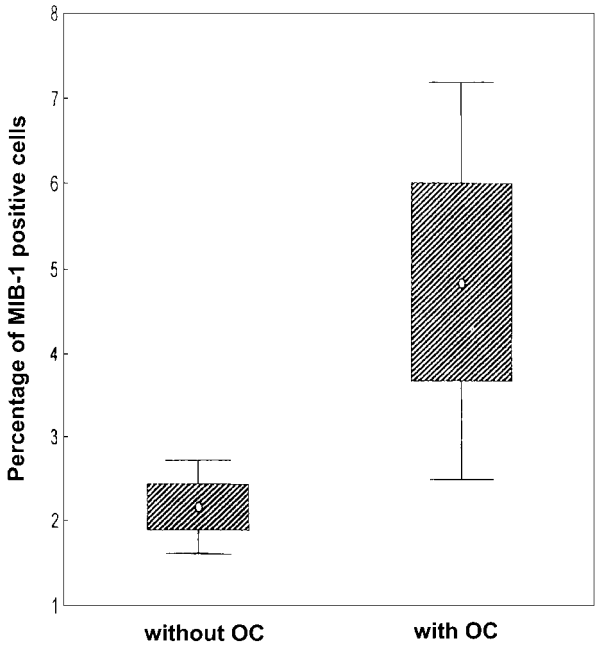

Figure 1. Proliferation in breast epithelial tissue expressed by percentage of MIB-1 positive cells in women with FNA biopsies evaluable for MIB-1 content, i.e. naturally cycling women ($n = 54$) and women using oral contraceptives ($n = 53$). Box-and-whisker plots representing the mean value with ± 1 SE of all data falling within the box. The 'whiskers' extend to ± 2 SE.

Table 1. Serum steroids, SHBG, IGF-1 and percentage of MIB-1 positive breast epithelial cells in women with FNA biopsies evaluable for MIB-1 content, that is 54 regularly menstruating healthy women and 53 women using combined oral contraceptives (OC)

	Non-users	OC users
Age, years	30.9 (19–45)	27.3 (18–50)**
% MIB positive cells	1.5 (0–8)	3.0 (0–50)*
A-4, nmol/l	6.4 ± 0.4	5.3 ± 0.3*
T, nmol/l	1.4 (0.6–3.8)	0.5 (0.1–1.8)***
SHBG, nmol/l	43.7 (15.6–85.9)	102 (19.5–434)***
fT, pmol/l	29 (11–76)	5 (1–14)***
IGF-1, μg/l	298 ± 11	308 ± 17
Progesterone, nmol/l	27.3 (2.6–77.8)	2.8 (1.0–5.0)***

Data are given as mean ± SEM or as median and range according to distribution. Significance of differences between groups are denoted by * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

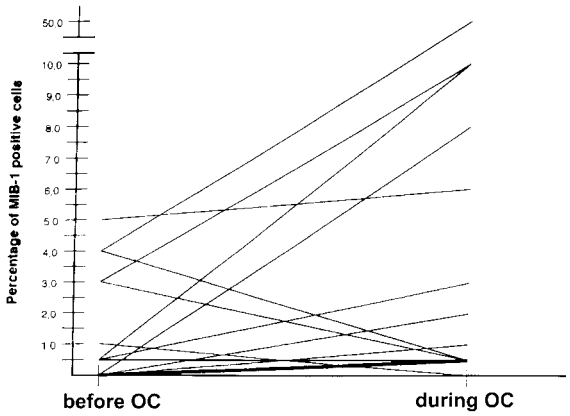

Figure 2. Percentage of MIB-1 positive cells in breast epithelial tissue before and after two months of oral contraceptive use ($n = 16$).

positive cells in the different treatment groups nor in the whole material.

Women using OCs had significantly lower serum levels of progesterone, A-4, T and fT and significantly higher SHBG levels than the non-users (Table 1). In non-users, breast epithelial proliferation showed a significant negative correlation to serum A-4 ($r_s = -0.29$, $p < 0.05$) and a significant positive correlation to progesterone at serum levels ≥ 30 nmol/l ($r_s = 0.43$, $p < 0.05$, $n = 22$). In OC users, breast epithelial proliferation showed a significant negative correlation to fT ($r_s = -0.38$, $p < 0.05$). There was no difference in breast epithelial proliferation between the 'second generation' OCs containing levonorgestrel ($n = 37$) and the 'third generation' OCs containing desogestrel ($n = 18$) (data not shown). The median serum concentration of levonorgestrel was 11.2 nmol/l (3.5–31.7). There was a significant positive correlation between breast epithelial proliferation and serum concentrations of levonorgestrel in those 37 women using OCs containing this progestogen ($r_s = 0.43$, $p = 0.02$).

Among the 26 women undergoing FNA biopsy before and after 2 months of OC use, 16 women had two evaluable samples (Figure 2). The percentage of MIB-1 positive cells before treatment was mean 1.4%, median 0.5% and range 0–5% and after 2 months of treatment with oral contraceptives containing ethinyl estradiol and levonorgestrel, the corresponding figures were 5.8%, 0.8% and 0–50% ($p = 0.055$). OC use significantly decreased serum levels of A-4, T, fT and IGF-I (data not shown).

Discussion

Oral contraceptives are used by numerous women but there are few studies on the influence of exogenous sex steroids on proliferation in normal breast tissue obtained from healthy women. Early efforts to assess proliferation in FNA biopsies were often hampered by low cellularity in cytologic smears. Here using FNA biopsy in combination with the cytospin technique, which uses the aspirated cells more effectively than the conventional smear technique, 81% of the obtained samples were evaluable for proliferation with the MIB-1 antibody.

We found a significantly higher breast epithelial proliferation in women using OCs as compared to non-users. The mean value for the percentage of MIB-1 positive cells of all the evaluable samples was 2.2% (range 0–8%) among naturally cycling women. In a previous study on breast epithelium during the menstrual cycle the corresponding value was 2.04% (range 0–6%) during the luteal phase [6]. Here among women using OCs, the mean value displayed a more than two-fold increase and there was also a marked individual variation. In some women as many as 40–50% of cells were stained positive for proliferation by the MIB-1 antibody. The increased proliferation and the individual variation was also apparent in those 16 women where proliferation was assessed before and after two months of OC use.

The hormonal regulation of proliferation in the normal breast is controversial and incompletely understood. While the breast is clearly sensitive to sex steroid action, the tissue concentrations of specific

receptors are comparatively low. In previous immunohistochemical studies we and others [13, 20] have found a positive receptor staining in only about 5–15% of examined cells. The present data are in agreement with findings in studies by Williams, et al. [7] and Andersson et al. [8] on breast tissue from women undergoing surgery for benign reasons. Women using OCs had a more pronounced proliferation and lower ER values compared to naturally cycling women whereas PR levels were unchanged or even increased.

A significant positive correlation was found between proliferation in breast tissue and circulating concentrations of levonorgestrel in women using OCs containing this synthetic progestogen. A significant positive correlation was also found in non-users between proliferation in breast tissue and serum progesterone at progesterone levels of 30 nmol/l and above, that is within the luteal phase range. Estrogen is generally accepted as a promoter of breast epithelial cell proliferation [21] and also thought to be involved in the development and growth of breast cancer. The effects of progesterone/progestogens are more complex and conflicting data have been presented [4, 22]. There is some evidence that progestogens might be mitogenic in breast tissue [22–24]. In the endometrium, progestogens counteract the proliferative effect of estrogen and both combined and progestogen-only contraceptives reduce the risk of endometrial cancer [25]. However, there are strong indications that the hormonal regulation of the normal breast is clearly different from that of the endometrium. In postmenopausal women the addition of progestogen during estrogen replacement will not reduce but may even increase the risk of breast cancer [26, 27]. In the large collaborative study progestogen-only contraceptives were shown to have an impact on breast cancer risk similar to that of combined OCs [1]. In naturally cycling women, the highest rate of breast epithelial proliferation was seen during the luteal phase [28] and proliferation was correlated to levels of progesterone [6]. In an animal model with surgically postmenopausal macaques, breast epithelial proliferation was more pronounced following combined estrogen/progestogen treatment than for treatment with estrogen alone [29, 30]. Furthermore, mammographic parenchymal density, which may reflect proliferation, was more pronounced during combined estrogen/progestogen replacement therapy than during treatment with estrogen alone [31, 32]. Together with the results of the present study all these findings strongly indicate progesterone/progestogen as a stimulatory factor for breast proliferation.

Serum levels of circulating androgens were markedly reduced during oral contraception which should partly reflect ovarian suppression. Significant negative correlations were found between breast proliferation and A-4 in the non-users and between breast proliferation and fT in OC users. Although A-4 is considered to be a weak androgen at the receptor level, it is reported to be a more important precursor than T for the formation of the terminal biologically active androgen 5 α-dihydrotestosterone in the target tissues in women. Serum concentrations of A-4 in women are higher than those of T and A-4 is far more bioavailable than T due to its lack of binding to SHBG [33]. Previously, testosterone has been found to suppress proliferation in breast epithelial cells [22, 34]. In clinical practice testosterone and androgenic compounds like danazol are often used to reduce mastalgia and may possibly reduce proliferation [35]. Tentatively reduced androgen levels could therefore also contribute to the increased proliferation seen during oral contraception.

From a clinical perspective increased breast epithelial proliferation during hormonal contraception should be regarded as an unwanted side effect. Increased proliferation could be potentially hazardous since high rates of cell turnover may increase the risk of neoplastic transformation [2]. Efforts should be made to define hormonal contraceptive regimens which minimize breast epithelial proliferation but maintain the many advantages, acceptability and overall safety of the method. The effects of different doses of estrogens and different doses and types of progestogens should be explored.

We also found a marked difference in breast epithelial proliferative response among women using the same OC regimen. The factors which regulate this apparent difference in individual sensitivity to exogenous steroids are at present unknown. Differences in body composition, absorption and pharmacokinetics of exogenous steroids like levonorgestrel, but also local factors like enzymatic activity within the breast may be important. In fact, the increased risk for breast cancer during long-term HRT seems to be primarily related to women with a low to normal BMI [26]. Means to identify those women with the most pronounced proliferative response during OC use should be developed. At present no methods for clinical surveillance of the breast of women during hormonal treatment are available and methodological development in this field is needed. It should be clarified to

what extent increased proliferation is related to the risk of breast cancer in women using oral contraceptives.

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