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Influences of percutaneous administration of estradiol and progesterone on human breast epithelial cell cycle in vivo*†

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Objective: To study the effect of E_2 and P on the epithelial cell cycle of normal human breast in vivo.

Design: Double-blind, randomized study. Topical application to the breast of a gel containing either a placebo, E_2, P, or a combination of E_2 and P, daily, during the 10 to 13 days preceding breast surgery.

Patients: Forty premenopausal women undergoing breast surgery for the removal of a lump.

Main Outcome measures. Plasma and breast tissue concentrations of E_2 and P. Epithelial cell cycle evaluated in normal breast tissue areas by counting mitoses and proliferating cell nuclear antigen immunostaining quantitative analyses.

Results: Increased E_2 concentration increases the number of cycling epithelial cells. Increased P concentration significantly decreases the number of cycling epithelial cells.

Conclusion: Exposure to P for 10 to 13 days reduces E_2-induced proliferation of normal breast epithelial cells in vivo. Fertil Steril 1995;63:785–91

Key Words: Human, in vivo, normal breast tissue, P, E_2, percutaneous administration, steroid concentration, proliferation markers, mitotic index, PCNA

Most research investigation programs on the natural history of breast disease focus on breast cancer cells between in situ and metastatic tumors. Little is known about the basic physiology of breast tissue and the early changes occuring between normal epithelium to hyperplasia. However, understanding the regulation and deregulation of mitotic activity in normal breast epithelial cells is an important endpoint, because epithelial hyperplasia is one of the major risk factors for breast cancer (1). Although there is a general consensus that 17β-E_2 even in physiological concentrations increases the mitogenic activity of epithelial cells, the influence of P has been debated for decades (2–6).

Progesterone is thought to either increase, decrease, or have no effect on mitotic activity and proliferation in breast epithelial cells. It may induce, reduce, or have no effect on hyperplastic lesions, and it may prevent or increase breast cancer risk during long-term use (2–4). Some studies call either for restrictions or widespread use of progestogens

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in contraception or in hormone replacement therapy (2, 5). These authors believe that spontaneous ovulatory cycles may either prevent or alternatively increase breast cancer risk (4, 6). Therefore, the many critical decisions required today are based on insufficient and conflicting data regarding P influence on normal epithelial breast cells.

In vitro studies generally show that P decreases the mitotic activity of normal human breast epithelial cells (7) and also partially inhibits the estrogen-induced proliferative response in most human cancerous cell lines (8). However, these data are not entirely convincing because the actual P influence may be different in vivo. The many complex interactions among myoepithelial, stromal, and adipose cells cannot be reproduced adequately in vitro (9).

In vivo the maximal proliferative rate of breast epithelium surgically biopsied has been reported variously to occur either in the early follicular phase (10), early luteal phase (11), or in the late luteal phase (3, 12), the latter results suggesting a mitogenic activity for P. None of these inconsistent studies has attempted to correlate the observed high interindividual variation in mitotic activity with individual P and E_2 concentrations in plasma and breast tissue at the time of biopsy. Patients submitted to general anesthesia for breast surgery, even for benign indications, are more likely to experience stress-induced disturbances in their P and E_2 ovarian secretion during the luteal phase and to have lower P concentrations on any given day of their late luteal phase (13, 14). One study conducted in women autopsied within 24 hours after sudden unexpected death showed that the drop in mitotic activity occurs approximately 8 days later in breast epithelium than in endometrium of the same subjects (12). These substantial differences between the endometrial and breast response to the same sex steroid plasma levels suggest fundamental differences in nature of or dynamism of the responses or both. Progesterone suppresses E_2 receptors in both endometrial and breast epithelial cells but does not seem to downregulate P receptors in breast epithelial cells (15, 16). Only one in vivo study has tried to induce stable breast tissue concentrations of either E_2 or P, during the days preceding surgery (14). This study has shown that sustained levels of P in breast tissue maintained for >10 days tend to decrease mitotic activity in the normal breast epithelial cells. The present study is an attempt to investigate the respective influences of E_2 and P on normal human breast epithelium in vivo.

MATERIALS AND METHODS

Patients

Forty premenopausal patients were enrolled in this study. They were between 18 and 45 years of age, had regular menstrual cycles, and had not taken any estrogen or progestin for at least 2 months. They were undergoing breast surgery for removal of a suspicious although presumably benign lump. The study design was approved by the Institutional Review Board of our University and a written informed consent was obtained for each woman before treatment.

Study Design

Surgery was scheduled between the 11th and 13th day of the menstrual cycle, before the presumed date of ovulation and the beginning of endogenous production of P. The subjects were assigned at random to receive a hydro-alcoholic gel (2.5 g/d) containing either a placebo or 25 mg of P, or 1.5 mg of E_2 or a combination of E_2 (1.5 mg) plus P (25 mg; Besins-Iscovesco Laboratory, Paris, France).

The gel was applied daily on the breast scheduled for surgery, from the 1st day of the menstrual cycle and during the 11 to 13 days preceding surgery. At time of surgery, a blood sample was taken for E_2 and P plasma level measurements by specific RIAs described previously (14).

Two samples of breast tissue (approximately 500 mg each) were taken in a macroscopically normal area at least 1 cm away from the lump. The first sample was stored at −20°C and processed for the measurement of E_2 and P tissue concentrations. The second sample was stored immediately in formalin and used for measurements of the cell cycle markers. Pathological tissues were sent to the pathologist for histologic diagnosis according to usual procedure.

Steroid Tissue Assays

Steroid extraction from the breast tissue was performed as follows (all manipulations were carried out at 0°C). Briefly, breast tissue (500 mg) was homogenized in water with a polytron. Radioactive tracer steroids were added to the mixture for calculation of the extraction rate. Steroids were then extracted twice with 15 mL of ether and then evaporated under nitrogen. One milliliter of 70% methanol in water was added to the residue and stored overnight at −20°C for deffating.

The mixture was centrifuged for 30 minutes (3,000 × *g*) at −5°C. A supernatant aliquot (0.8 mL) was taken and evaporated. If the residue was not limpid, it was dissolved in ether; the other phase was evaporated and the residue dissolved in dichloromethane-methanol. It was then applied to a Sephadex LH 20 column (Pharmacia, St. Quentin, France) in dichloromethane-methanol (90:10). Fractions were collected and radioactivity was determined in all fractions, after which pools of P and E_2 were made. The E_2 concentration was calculated directly by RIA. For P, the residue was dissolved in 0.5 mL of saturated isooctane with 10% ethyleneglycol. Chromatography on a celite column was prepared: pure P fractions were evaporated and then dissolved in 2 mL of ethanol for final RIA as described previously (14).

Mitotic Index (MI)

Normal breast tissue preparations were stained by the pararosaniline Feulgen-Schiff techniques (17). Cells in the intralobular and in the large interlobular ducts were counted, as well as the acinar cells. The term "acinus" is used to describe the terminal units seen within the breast during the resting stage. "Ductal cells" are the epithelial, luminal, and myoepithelial cells that line the intralobular and extralobular ducts. The MI was defined as the number of mitotic figures divided by the total number of cells counted expressed per 1,000 cells. Up to 5,000 cells were counted in six nonsequential sections; the whole section was scanned at ×40.

The proliferating cell nuclear antigen (PCNA) also known as cyclin, is an acidic nuclear protein with an apparent molecular mass of 36 kd. Monoclonal antibodies raised against PCNA (18) have been shown to correlate with the proliferative compartment in conventionally fixed and processes normal human tissues. Proliferating cell nuclear antigen was analyzed with the murine immunoglobulin G 2ak monoclonal antibody PC-10 anti-PCNA antibody developed by Waseem et al. (18). Immunohistochemical assays were performed in conventionally formalin-fixed, paraffin-embedded sections. Sections were cut at room temperature (not heated to assist adherence to glass slides), mounted on poly-L-lysine—coated glass slides, and air-dried overnight at room temperature. Sections were dewaxed and rehydrated and then treated for 1 hour with 3% H_2O_2 in absolute methanol, to block endogenous peroxidase activity. Sections then were incubated in 0.5% tween, 2% bovine serum albumin in phosphate-buffered saline for 30 minutes. Immunostaining was carried out using a streptavidin biotin peroxidase substrate. The primary antibody was diluted 1:100 and incubated overnight. 3-Amino-9-ethylcarbazol was employed as a chromogen. As positive control for PC-10, a lymphoid tissue section was included.

Immunohistochemical Analysis

The immunostaining quantitative analysis was done by means of a computer-assisted image processor (19) (Système d'Analyse Microphotométrique à Balayage Automatique; Samba-Alcatel, Grenoble, France). This microcomputer-based system is configurated with a standard microscope (Polyvar; Richert Jung, Cambridge, United Kingdom), a color video camera (Sony Co., Kanagawa-Ken, Japan), and a 80286 computer (V286; Victor, Stockholm, Sweden). The intensity and distribution of PCNA labeling in hematoxylin-counterstained tissue sections was quantified. Proliferating cell nuclear antigen was analyzed as a false red color, whereas counterstained cells were analyzed as a false blue color. For each preparation, optical density (OD) thresholds were determined using real microscopic images of the analyzed field as reference. Measurements of immunostaining were performed at ×25. Twenty fields were analyzed for each section. Indices of stained nuclear surface and immunostaining OD were expressed in arbitrary units. Controls for immunostaining quantitative analysis reproducibility were carried out by [1] comparison to iterative measurements done on the same preparations and [2] comparison to measurements completed on five sequential sections of the same specimen. Evaluation of tissue variations in PCNA immunostaining quantitative analysis was carried out by comparing the immunostaining OD measurements of 20 fields taken from each of 6 nonconsecutive sections from each normal specimen.

The intensity of PCNA immunoreactivity displayed by actively proliferating cells in lymph nodes (centroblast) served as a reference for thresholds positive staining in breast tissues.

Statistical Analysis

Statistical analyses were performed using the Kruskal Wallis nonparametric test of variance among the four treatment groups, separately for each parameter. When the null hypothesis (the four groups are identical) was rejected at the level 0.05, a multiple comparison procedure (20) was used to de-

Table 1 Intraglandular Steroid Concentration*

Treatment	Placebo (n = 8)	P (n = 7)	E_2 (n = 9)	E_2 + P (n = 9)
P (ng/g)†	0.6 ± 0.3	66 ± 120‡	2.1 ± 3.8§	41.2 ± 75.2‡‖
E_2 (ng/g)¶	0.5 ± 0.4	0.5 ± 0.7	91.0 ± 232.7‡§	35.5 ± 69.6‡§

* Values are means ± SD.
† Conversion factor to SI unit, 3.180.
‡ $P < 0.05$ versus placebo.
§ $P < 0.05$ versus P group.
‖ $P < 0.05$ versus E_2 group.
¶ Conversion factor to SI unit, 3.671.

termine which pairs of groups differed. The same level of statistical significance (0.05) was used in the whole procedure. Relation between the two proliferation markers, mitosis, and PCNA were analyzed by a Spearman correlation coefficient.

RESULTS

Patients and Treatments

Thirty-four of forty patients initially enrolled effectively completed the study. Six patients dropped out before surgery. One patient was excluded because of early ovulation with a plasma P level of 12 ng/mL (conversion factor to SI unit, 3.180) on day 12 of her menstrual cycle. Among the remaining 33 patients, 8 patients received a placebo, 7 received the P gel, 9 patients received the E_2 gel, and 9 received the E_2 + P gel.

Plasma Levels

The mean P plasma levels were consistently <1 ng/mL (conversion factor to SI unit, 3.18) in each treatment group. Estradiol plasma levels were within the physiologic range of normal menstrual cycle in all groups (mean range 57 ± 31 to 223 ± 99 pg/mL [conversion factor to SI unit, 3.67]). Estradiol plasma values were not significantly different in any treatment group when considered separately. However, when E_2 plasma values of users of the two gel formulations containing E_2 (223 ± 99 pg/mL with E_2 gel and 122 ± 108 pg/mL with E_2 + P associated gel) were pooled and compared with those of users of gel containing no E_2 (57 ± 31 pg/mL with P gel and 95 ± 63 pg/mL with placebo gel), the difference reached statistical significance ($P < 0.05$).

Tissue Concentrations

The mean P tissue concentrations were 0.6 ± 0.3, 66 ± 120, 2.1 ± 3.8, and 41.2 ± 75.2 ng/g tissue in the placebo, P-, E_2-, and E_2 + P-treated groups, respectively. Progesterone tissue concentrations were significantly higher ($P < 0.05$) in the two groups treated with P (P and E_2 + P) than in the two other groups (placebo and E_2) (Table 1). The mean E_2 tissue concentrations were 0.5 ± 0.4, 0.5 ± 0.7, 91 ± 232, and 35 ± 69 ng/g tissue in the placebo, P-, E_2- and E_2 + P-treated groups, respectively. Estradiol tissue concentrations in group E_2 and E_2 + P were significantly higher than in the placebo or P gel group ($P < 0.05$).

Proliferation Markers

Mitotic index was significantly lower in the P group compared with the placebo group, whereas it was significantly higher in the E_2 group compared with the P group ($P < 0.05$). The MI in the E_2 + P group was comparable to the placebo group and was not significantly different from any other groups (Table 2).

The PCNA labeling index (LI) was 7.8% ± 4.8% in the placebo group, significantly higher (17.4% ± 6.4%) ($P < 0.05$) in the E_2 group and significantly lower ($P < 0.05$) in the P group (1.9% ± 0.8%). In comparison with the E_2 group PCNA LI was reduced significantly (6.5% ± 4.4%) ($P < 0.05$) in the E_2 + P group. A significant correlation between the MI and the PCNA LI ($P < 0.05$) with a correlation coefficient of 0.50 was found.

DISCUSSION

Despite the urgent need for many critical clinical decisions in current practice, precise and undisputed information concerning the influence of P on the normal human breast is still lacking. Specifically, it is not known whether or not P may influence epithelial proliferation and occurence of ductal hyperplasia. Epidemiologic and intervention studies have numerous biases including the use at different doses and durations of various synthetic

Table 2 Proliferation Markers in Normal Lobular Epithelial Cells*

Treatment	Placebo	P	E_2	E_2 + P
Mitosis per 1000 cells	0.51 ± 0.24	0.17 ± 0.19*	0.83 ± 0.42†	0.52 ± 0.42
PCNA (LI %)	7.8 ± 4.8	1.9 ± 0.8*	17.4 ± 6.4*†	6.5 ± 4.4‡

* Values are means ± SD.
† $P < 0.05$ versus placebo.
‡ $P < 0.05$ versus P group.
§ $P < 0.05$ versus E_2 group.

progestins (21). In vitro studies may be misleading because they do not reproduce adequately interactions between epithelium and stroma and because the other factors added to the in vitro milieu may not be present in vivo, thereby changing the outcome. The previously published conflicting results derived from human breast biopsies in vivo might be improved by a more careful evaluation of intensity and duration of hormonal stimulation at the time of biopsy and by a better identification of the cell cycle.

The present protocol derived from the Barrat et al. study (14) is likely to provide a more reliable understanding of the respective P and E_2 influences in comparison with previous studies.

In the present study daily topical breast application of a placebo, E_2, P, or E_2 + P gel for 10 to 13 days before surgery induced limited changes in plasma E_2 or P levels while producing markedly different levels of steroid accumulation within the breast tissue. In agreement with previous studies (13, 14) plasma P levels were not been changed by topical application of P gel to the breast. The applied dose is expected to deliver 2.5 mg of P to the breast tissue, approximately 10% of the entire ovarian production during the luteal phase. This delivered dose should not be enough to affect significantly serum concentrations. Daily application of 1.5 mg E_2 in a gel formulation on a relatively small skin surface area (approximately 200 cm^2) is expected to deliver <50 μg/d of E_2, a dose equivalent to approximately 50% of the mean endogenous production and responsible for the small but significant increase in E_2 plasma values in users of the E_2 or E_2 + P gel formulation. Irrespective of the plasma changes, breast tissue P concentrations, as expected, were significantly higher in the P- and E_2 + P-treated groups, and E_2 concentrations were significantly higher in the E_2- and E_2 + P-treated groups.

Traditionally, the growth fraction has been measured by autoradiography using radiolabeled thymidine or by evaluating the number of visible mitoses. The former method measures those cells in the S phase but is somewhat cumbersome and depends on many experimental factors.

Counting mitotic figures (Fig. 1) identifies only a very small percentage of the cycling cells, exclusively those in the short M phase (1 per 10,000 to 1 per 1,000 epithelial cells). This procedure requires careful examination of thousands of epithelial cells, and therefore produces a reduced quantity and quality of information with limited statistical value. An

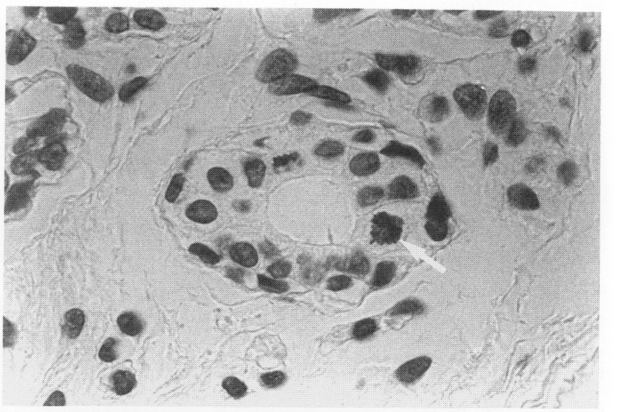

Figure 1 Mitotic figure in a normal breast epithelial cell (×100).

endogenous cell proliferation marker (PCNA), which is preserved during tissue processing for pathological studies, improves measurements of the mitotic growth fraction (Fig. 2). Proliferating cell nuclear antigen immunostaining identifies all cycling cells from the late G1 to G2M phase (1 per 100 to 1 per 5 epithelial cells). The interpretation of PCNA immunoreactivity in normal tissues has been reviewed widely (22, 23). Proliferating cell nuclear antigen is a reliable marker of cell proliferation in non-neoplastic tissues. The computerized detection of PCNA-labeled cells reduces subjectivity, improves reproducability, and eases the workload. To summarize, PCNA LI produces approximately 100 times more information than MI from the same limited number of epithelial cells available from the breast biopsy and therefore amplifies the validity of the cell cycle analysis. For example, differences in epithelial cell cycle between E_2 and E_2 + P groups reach statistical significance according to PCNA LI but not when using the simple counting of mitotic figures. Similarly, the differences between E_2 and placebo groups are significantly different by the PCNA LI method but not by MI.

Blinded evaluation of the MI of normal breast epithelial cells has shown significant variation according to ambient steroid concentrations. This index was lower in the P groups and higher in the E_2 group. These results are in agreement with the study of Barrat et al. (14). Adding P to E_2 tended to reduce the MI to the level seen in the placebo-treated group.

Computerized analysis of PCNA-labeled cells has provided for the first time strong confirmatory

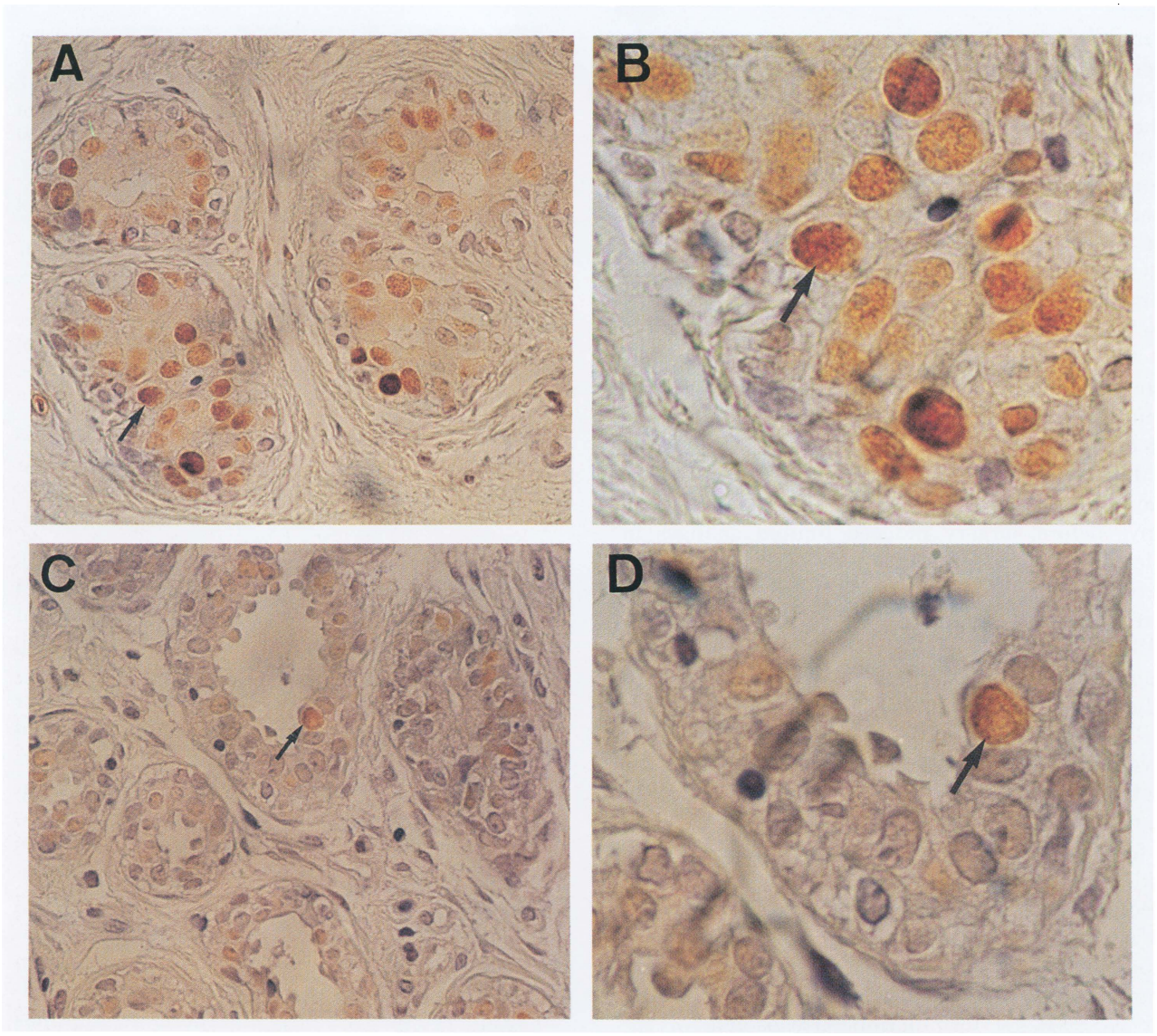

Figure 2 Proliferating cell nuclear antigen-labeling index in normal breast tissues was determined using a low threshold of reactivity. Proliferating cell nuclear antigen immunoreactivity displayed by active proliferating cells in positive controls was retained as threshold. **A** (×60) and **B** (×100) show the immunoreactivity observed in tissue from a E_2-treated patient (PCNA LI: 19.4%). **C** (×60) and **D** (×100) show the PCNA immunostaining observed in tissue from a P-treated patient (PCNA LI: 1.1%). Arrows indicate the intensity of PCNA immunostaining retained as threshold.

results with significant correlation between MI and PCNA-labeling index. Specifically adding P to E_2 significantly reduced the proliferative effect of E_2 alone. The present data shows that in vivo, 10 to 13 days of P exposure decreases the growth fraction of normal epithelial cells in the breast of premenopausal women. These data do not provide information about the effects of shorter duration of P exposure, which may first stimulate before inhibiting epithelial proliferation (8). Standard teaching on breast physiology includes a short-term role for P in the stimulation of alveolar cell proliferation in the early luteal phase (i.e., after few days of P exposure). These classical data do not exclude the possibility of an inhibitory response in breast epithelium proliferation to longer P treatment. They also do not exclude the possibility of a delay in breast response in comparison with endometrium. In the present study we evaluated the more sustained effect of P lasting 10 to 13 days.

Two studies have suggested that endogenous P secretion suppresses E_2 receptors in breast epithe-

lial cells as is also the case in endometrium, but does not down regulate P receptors (15, 16). Suppression of E_2 receptors is consistent with an antiestrogenic effect, but the consequence of persisting P receptors is unknown. In studies showing stimulation of human breast cancer cells by very high concentrations of some synthetic progestins, a cross reactivity with E_2 receptors also has been shown, but activation of P receptors has been excluded as the growth stimulatory mechanism (24, 25). The present data strongly support the concept that physiologic secretion of P during a normal luteal phase favorably influences the control of the human breast epithelial cell cycle. It also suggests that P or related drugs may have a therapeutic value to prevent breast epithelial hyperplasia when used $\geq$10 days per month at approximate substitutive doses.

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REFERENCES

1. Page DL, Dupont WD. Premalignant conditions and markers of elevated risk in the breast and their management. Surg Clin N Am 1990;70:831–51.
2. Key T, Pike M. The role of estrogens and progestogens in the epidemiology and prevention of breast cancer. Eur J Cancer Clin Oncol 1988;24:29–43.
3. Going J, Anderson T, Battersby S. Proliferative and secretory activity in human breast during natural and artificial menstrual cycle. Am J Pathol 1988;130:193–204.
4. Vorherr H. Fibrocystic breast disease: pathophysiology, pathomorphology, clinical picture, and management. Am J Obstet Gynecol 1986;154:161–79.
5. Don Gambrell R. Hormone replacement therapy and breast cancer. Maturitas 1987;9:123–33.
6. Henderson B, Ross R, Judd H, Krailo M, Pike M. Do regular ovulatory cycles increase breast cancer risk? Cancer 1985;56:1206–8.
7. Gompel A, Malet C, Spritzer P. Progestin effect on cell proliferation and 17β-hydroxysteroid dehydrogenase activity in normal human breast cells in culture. J Clin Endocrinol Metab 1986;63:1174–80.
8. Clarke CL, Sutherland RL. Progestin regulation of cellular proliferation. Endocrine Rev 1990;11:266–301.
9. Coldham NG, James VHT. A possible mechanism for increased breast cell proliferation by progestins through increased reductive 17β-hydroxysteroid dehydrogenase activity. Int J Cancer 1990;45:174–8.
10. Vogel PM, Georgiade NG, Fetter BF. The correlation of histologic changes in the human breast with the menstrual cycle. Am J Pathol 1981;104:23–34.
11. Potten CS, Watson RJ, Williams GT. The effect of age and menstrual cycle upon proliferative activity of the normal human breast. Br J Cancer 1988;58:163–70.
12. Longacre TA, Bartow SA. A correlative morphologic study of human breast and endometrium in the menstrual cycle. Am J Surg Pathol 1986;10:382–93.
13. De Boever J, Verheugen C, Van Maele G. Steroid concentrations in serum, glandular breast tissue, and breast cyst fluid of control and P-treated patients. In: Angeli A, editor. Endocrinology of cystic breast disease. New York: Raven Press, 1983:93–9.
14. Barrat J, De Lignieres B, Marpeau L. Effet in vivo de l'administration locale de progestèrone sur l'activité mitotique des galactophores humains. J Gynecol Obstet Biol Reprod 1990;19:269–74.
15. Battersby S, Robertson BJ, Anderson TJ, King RJ, McPherson K. Influence of menstrual cycle, parity and oral contraceptive use on steroid hormone receptors in normal breast. Br J Cancer 1992;41:601–7.
16. Soderqvist G, Von Schoultz B, Tani E, Skoog L. Estrogen and P receptor content in breast epithelial cells from healthy women during the menstrual cycle. Am J Obstet Gynecol 1993;168:874–9.
17. Kenji M, Yoshikazu K, Sadamu N, Yoshihiro U. Automated Feulgen's reaction in autoscreening. J Jap Soc Clin Cytol 1971;10:148–54.
18. Waseem NH, Lane DP. Monoclonal antibody analysis of the proliferating cell nuclear antigen/PCNA: structural conservation and the detection of a nucleolar form. J Cell Sci 1990;96:121–9.
19. Opferman M, Brugal G, Vassilakos P. Cytometry of breast carcinoma: significance of ploidy balance and proliferation index. Cytometry 1987;8:217–24.
20. Conover WJ. Practical non parametric statistics. New York: J Wiley, 1980:229–32.
21. Staffa JA, Newschaffer CJ, Jones JK, Miller V. Progestins and breast cancer: an epidemiologic review. Fertil Steril 1992;57:473–91.
22. Dietrich DR. Toxicological and pathological applications of Proliferating cell nuclear antigen (PCNA), a novel endogenous marker for cell proliferation. Crit Rev Toxicol 1993;23:77–109.
23. Bravo R, Frank R, Blundell PA, MacDonald-Bravo H. Cyclin/PCNA is the auxiliary protein of DNA polymerase-d. Nature 1987;326:515–7.
24. Van der Burg B, Kalkhoven E, Isbrucker L, de Laat SW. Effects of progestins on the proliferation of estrogen dependant human breast cancer cells under growth factor-defined conditions. J Steroid Biochem 1992;42:457–65.
25. Jeng MH, Parker CJ, Jordan VC. Estrogenic potential of progestins in oral contraceptives to stimulate human breast cancer cell proliferation. Cancer Res 1992;52:6539–46.