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Estradiol and progesterone regulate the proliferation of human breast epithelial cells

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Objective: To study the effects of estradiol and progesterone on the proliferation of normal human breast epithelial cells in vivo.

Design: Double-blind randomized study.

Setting: Departments of gynecology and of cell biology at a university hospital.

Patient(s): Forty postmenopausal women with untreated menopause and documented plasma FSH levels of >30 mIU/mL and estradiol levels of <20 pg/mL.

Intervention(s): Daily topical application to both breasts of a gel containing a placebo, estradiol, progesterone, or a combination of estradiol and progesterone during the 14 days preceding esthetic breast surgery or excision of a benign lesion.

Main Outcome Measure(s): Plasma and breast tissue concentrations of estradiol and progesterone. Epithelial cell cycles were evaluated in normal breast tissue by counting mitoses and performing quantitative proliferating cell nuclear antigen immunolabeling analyses.

Result(s): Increasing the estradiol concentration enhanced the number of cycling epithelial cells, whereas increasing the progesterone concentration significantly limited the number of cycling epithelial cells.

Conclusion(s): Exposure to progesterone for 14 days reduced the estradiol-induced proliferation of normal breast epithelial cells in vivo. (Fertil Steril® 1998;69:963–9. ©1998 by American Society for Reproductive Medicine.)

Key Words: Human, in vivo, normal breast, progesterone, estradiol percutaneous administration, steroid concentration, proliferation markers, proliferating cell nuclear antigen, mitotic index, DNA ploidy

Because breast cancers arise in the epithelial cells of the terminal ductal lobular unit, understanding the endocrine regulation of their proliferation is important. 17β-Estradiol, at physiologic concentrations, is known to stimulate the proliferation of normal breast epithelial cells, whereas the influence of progesterone has been debated for two decades.

Depending on the models used (animal or human, in vitro or in vivo studies), the timing of progesterone administration, and the method of evaluation used (mitotic index, proliferating cell nuclear antigen, K_{i67}, or [^{3}H]-thymidine labeling), progesterone is reported to stimulate, reduce, or have no effect on the mitotic activity and proliferation of breast epithelial cells (1–3). These same studies suggest that progesterone and/or synthetic progestins also can stimulate, reduce, or have no effect on hyperplastic lesions (1–3). Colditz et al. (4) showed that the addition of progestins to estrogen therapy did not reduce the risk of breast cancer among postmenopausal women. On the other hand, Gambrell (5) demonstrated that the incidence of breast cancer was significantly lower in women treated with estrogen and progestin than in untreated or estrogen-treated postmenopausal women.

Similarly, Risch and Howe (6) and Stanford et al. (7), in large cohort studies, also recently reported that progestins diminished the estrogen-associated risk of breast cancer. Therefore, the therapeutic indications of progesterone treatment or its avoidance are based on insuf-

ficient and controversial epidemiologic evidence and on a lack of adequately controlled data regarding the influence of progesterone on normal breast epithelium.

At the time of surgery, normal breast tissue adjacent to fibroadenomas has been used to histologically assess the proliferative activity in the terminal ductal lobular unit. Several studies indicated optimum proliferation during the luteal (progestogenic) phase of the menstrual cycle (2, 8–11). The data were interpreted as an indication that progesterone stimulates the proliferation of normal human terminal ductal lobular unit epithelial cells. However, the samples had been obtained from women whose endocrine profiles (plasma and tissue estradiol and progesterone concentrations) had not been characterized.

However, when terminal ductal lobular unit cells are excised and grown in hormonally treated immunodeficient mice, a different picture emerges: estradiol stimulates the growth of the epithelial cells and progestins have no effect (12). Epithelial cells cultured from normal breast tissue also are stimulated by estradiol but not by progestin (13) or progesterone (14).

A recent in vivo, double-blind, randomized trial in premenopausal women demonstrated that the percutaneous administration of estradiol and/or progesterone to the breast for 10–13 days before surgery for the removal of a mammary lump had opposite effects. Increased tissue estradiol concentrations stimulated the growth of cycling epithelial cells, whereas progesterone administration decreased the number of proliferating epithelial cells (15).

When premenopausal women with spontaneous endocrine ovarian activity are hospitalized and undergo general anesthesia for breast surgery, they are more likely to experience stress-induced disturbances in their endogenous progesterone and estradiol ovarian secretions that might interfere with the exogenously applied steroids. We therefore conducted a double-blind, randomized clinical trial in 40 postmenopausal women who had not received any hormone replacement therapy (HRT) to determine precisely the effects of estradiol, progesterone, and combined estradiol and progesterone on breast epithelial cell proliferation.

MATERIALS AND METHODS

Patients

Forty postmenopausal women with documented untreated menopause (FSH level of >30 mIU/mL; estradiol level of <20 pg/mL) were enrolled in this study. Previous HRT had to have been withdrawn for at least 12 weeks. The women were assigned randomly to one of four treatment groups, each of which received a hydroalcoholic gel (2.5 g/d) containing one of the following drugs or drug combinations: [1] estradiol (1.5 mg/d); [2] progesterone (25 mg/d); [3] estradiol (1.5 mg/d) and progesterone (25 mg/d); or [4] placebo. Each gel formulation was applied daily to both breasts for 14 days before plastic surgery or excision of a presumably benign lesion. The steroids were applied percutaneously to more closely mimic the physiologic effects of natural progesterone.

Neither the intramuscular injection nor the oral administration of progesterone reproduce exactly the biologic effects of endogenous progesterone on its classic targets, such as the breast or endometrium. This could be the consequence of cyclic and large interindividual variations in plasma concentrations after IM injections or of the associated metabolites after the first-pass effect through the liver (16). The vaginal administration of progesterone induces a secretory transformation of the endometrium because of its local accumulation (16). Its systemic effect at sites distant from the vagina is not documented.

In contrast, three previous studies have documented the possibility of raising the tissue concentration of progesterone to the physiologic levels reached during a normal luteal phase by percutaneous administration of this steroid in a hydroalcoholic gel (15, 17, 18). After diffusion through the skin, this steroid becomes evenly distributed, probably by the hematogenous route in the mammary tissue independent of the distance from the skin surface (17).

Estradiol also was applied percutaneously to the breast in accordance with the design of the study (double-blind randomization). The study design was approved by the university ethics committee, and informed consent was obtained from each participant.

Study Design

Surgery was performed on study day 15. During surgery, a blood sample was taken for determination of estradiol and progesterone plasma concentrations with the use of specific RIAs described previously (15). Two samples of normal breast tissue (approximately 500 mg each) were taken in a normal area of the breast at least 5 cm away from any lesion. The first sample was stored at −20°C and processed for measurement of estradiol and progesterone tissue concentrations. The second sample was fixed in formalin and used to quantitate proliferating cell nuclear antigen expression according to a previously described protocol (15) and to evaluate DNA ploidy.

The cycle marker proliferating cell nuclear antigen, also known as cyclin, is an acidic nuclear protein with an apparent molecular mass of 36 kd. Monoclonal antibody PC-10 anti–proliferating cell nuclear antigen antibody, made by Waseem and Lane (19), was used in immunohistochemical labeling assays on formalin-fixed, paraffin-embedded sections, as previously described in detail (15).

Automated quantitation of the immunolabeling was obtained with a computer-assisted image processor (SAMBA; Alcatel, Grenoble, France), which determined the background optical density thresholds for each preparation and subtracted them from the experimental readings, as previously reported (15). At

TABLE 1

Demographic characteristics of 40 women who received placebo, progesterone, estradiol, or estradiol and progesterone.

Demographic characteristic	Treatment group			
	Placebo (n = 10)	Progesterone (n = 13)	Estradiol (n = 10)	Estradiol + progesterone (n = 7)
Age (y)				
Mean ± SD	62.7 ± 9.9	63.3 ± 8.9	59.3 ± 5.3	58.1 ± 6.7
Median	66	64	59.5	59
Range	47–77	51–80	50–67	50–69
Weight (kg)				
Mean ± SD	66.4 ± 4.9	62.2 ± 9.2	66.2 ± 6.5	71.6 ± 13.4
Median	67.5	60	65	67
Range	55–73	48–80	57–79	57–92
Height (cm)				
Mean ± SD	163.8 ± 3.5	162.2 ± 2.8	163.4 ± 3.5	164.5 ± 5.0
Median	164.5	162	164.5	164
Range	156–168	159–168	158–169	159–172

least 2,000 nuclei from each tissue sample were evaluated in 20 fields containing terminal ductal lobular unit cells from each of six nonconsecutive sections from each normal specimen. Appropriate positive and negative controls were included. All analyses were performed blindly before the trial code was opened.

Steroid Tissue Analysis

Steroids were extracted from the breast tissues 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 to enable calculation of the extraction yield. Steroids then were extracted twice with 15 mL of ether and evaporated under nitrogen. One milliliter of 70% methanol in water was added to the residue and stored overnight at −20°C for defatting.

The mixture was centrifuged for 30 minutes (3,000 × *g*) at −5°C. A supernatant aliquot (0.8 mL) was taken and evaporated. When the residue was not limpid, it was dissolved in ether; the other phase was evaporated, and the residue was dissolved in dichloromethane-methanol and then applied to a Sephadex LH 20 column (Pharmacia, St.-Quentin, France) in dichloromethane-methanol (90/10 vol/vol). Fractions were collected and the radioactivity was measured in all fractions; the estradiol and progesterone pools were separated. The estradiol concentration was calculated directly by RIA. For progesterone, the residue was dissolved in 0.5 mL of saturated isooctane with 10% ethylene glycol and subjected to celite column chromatography. Purified progesterone fractions were evaporated and then dissolved in 2 mL of ethanol for RIA as described previously (18).

Mitotic Index

Cells in the terminal ductal lobular unit were counted. The mitotic index was defined as the number of mitotic figures divided by the total number of epithelial cells counted (at least 10,000) in six nonconsecutive sections expressed per 1,000 cells. All sections were scanned at ×40 magnification.

Statistical Analyses

Statistical analysis was performed with the use of the Kruskal-Wallis nonparametric test of variance among the four treatment groups. When the null hypothesis (the four groups are identical) was rejected at the 0.05 level, a multiple comparison procedure was used to determine statistical significance ($P<0.05$ by Student's *t*-test).

RESULTS

Patients and Treatment

Forty of the 44 women initially enrolled completed the study. Four patients were excluded because their baseline estradiol and FSH levels demonstrated persistent ovarian activity. Among the 40 remaining volunteers, 10 received a placebo, 13 received the progesterone gel, 10 received the estradiol gel, and 7 received the combined estradiol and progesterone gel (Table 1). No statistically significant differences were found between the mean ages, heights, and weights of the participants in the four groups.

Plasma Hormone Concentrations

Table 2 shows the plasma estradiol and progesterone levels at the time of surgery in all 40 women. A statistically significant ($P<0.001$) difference between the mean plasma progesterone concentrations (after logarithmic conversion) of the treatment groups was observed. The mean progesterone concentration was significantly higher in the groups treated with the progesterone gel and the combined estradiol and progesterone gel than in the

TABLE 2

Plasma and tissue hormone concentrations for 40 women who received placebo, progesterone, estradiol, or combined estradiol and progesterone.

Hormone specimen	Treatment group				*P* value*
	Placebo	Progesterone	Estradiol	Estradiol + progesterone	
Plasma progesterone (pg/mL)					
No. of patients	8	13	10	7	
Mean ± SD	281.4 ± 221.2	1265.6 ± 281.3	300.2 ± 108.1	952.1 ± 302.3	<0.001
Median	194.5	1,432	287.5	830	
Range	126–863	830–1667	180–454	620–1,400	
Plasma estradiol (pg/mL)					
No. of patients	8	13	10	7	
Mean ± SD	31.6 ± 11.0	30.8 ± 8.6	73.7 ± 20.6	75.4 ± 66.8	<0.001
Median	32.5	31	71.5	59	
Range	14–45	19–46	47–120	32–224	
Tissue progesterone (ng/g)					
No. of patients	10	13	10	7	
Mean ± SD	3.7 ± 2.4	17.8 ± 20.0	4.5 ± 1.8	11.2 ± 8.2	0.056
Median	3	12.2	4.2	9.1	
Range	1.6–9.8	5.4–82.0	2.4–7.3	4.0–29.1	
Tissue estradiol (pg/g)					
No. of patients	10	10	9	7	
Mean ± SD	278.9 ± 104.0	199.1 ± 67.8	659 ± 462.7	565.7 ± 355.4	0.010
Median	283	207.5	470	588	
Range	76–435	100–320	208–1,557	112–1,096	

* The differences between the means among the four groups were statistically significant.

groups treated with the estradiol gel and the placebo ($P < 0.001$) (Table 3). The group that received estradiol (estradiol gel or combined estradiol and progesterone gel) had significantly higher mean plasma estradiol levels ($P < 0.001$) than the groups that received progesterone gel or placebo.

TABLE 3

Statistical comparisons (Student's *t*-test) of hormone concentrations and histologic proliferation markers among the study groups.

Parameter	Treatment group	*P* value for indicated treatment group		
		Placebo	Progesterone	Estradiol + progesterone
Hormone concentration				
Plasma progesterone (pg/mL)	Estradiol	NS (0.488)	<0.001	<0.001
	Progesterone	<0.001		
	Estradiol + progesterone	<0.001	NS (0.119)	
Plasma estradiol (pg/mL)	Estradiol	<0.001	<0.001	NS (0.745)
	Progesterone	NS (0.859)		
	Estradiol + progesterone	<0.001	<0.001	
Tissue estradiol (pg/g)	Estradiol	0.019	0.002	NS (0.396)
	Progesterone	NS (0.335)		
	Estradiol + progesterone	NS (0.171)	S (0.031)	
Proliferation marker				
Proliferating cell nuclear antigen index (%)	Estradiol	<0.001	<0.001	<0.001
	Progesterone	<0.001		
	Estradiol + progesterone	0.003	NS (0.112)	
Mitotic index per 1,000 cells	Estradiol	<0.01	<0.05	<0.05
	Progesterone	NS		
	Estradiol + progesterone	NS	NS	

Note: NS = not significant; S = significant.

TABLE 4

Histologic proliferation markers for 40 women who received placebo, progesterone, estradiol, or combined estradiol and progesterone.

Marker	Treatment group: Placebo (n = 10)	Progesterone (n = 13)	Estradiol (n = 10)	Estradiol + progesterone (n = 7)	*P* value
Proliferating cell nuclear antigen labeling index (%)					
Mean ± SD	0.1 ± 0.1	1.5 ± 0.6	11.5 ± 2.3	1.3 ± 1.1	<0.001*
Median	0.1	1.3	10.6	1	
Range	0–0.3	0.9–3.0	8.6–5.4	0–3.2	
Mitotic index per 1,000 cells					
Mean ± SD	0.15 ± 0.20	0.19 ± 0.25	0.6 ± 0.2	0.2 ± 0.15	
Median	0.14	0.15	0.58	0.18	
Range	0–0.35	0.1–0.6	0.30–0.95	0–0.5	

* Statistically significant differences between the means among the four groups.

Tissue

The mean tissue estradiol concentrations were significantly higher in the groups that received estradiol gel and combined estradiol and progesterone gel than in the groups that received progesterone gel or placebo ($P<0.01$) (Table 2). Because of wide interindividual variations, progesterone tissue concentrations did not vary significantly among the groups (Table 2).

Histologic Proliferation Markers

The mean proliferating cell nuclear antigen labeling index was highest (11.5% ± 2.3%) in the estradiol group (Table 4). The mean proliferating cell nuclear antigen labeling index in the progesterone group was significantly lower than that in the estradiol group but significantly higher than that in the placebo group. Finally, the mean proliferating cell nuclear antigen labeling index in the combined estradiol and progesterone group was significantly lower than that in the estradiol group, with a mean level comparable to that of the progesterone group (Tables 3 and 4).

The mitotic index confirmed the absence of breast epithelial cell proliferation in postmenopausal women who did not receive HRT. Estradiol induced growth of the terminal ductal lobular unit (mitotic index = 0.6 ± 0.2), whereas the mitotic index in the progesterone and combined estradiol and progesterone groups was not significantly different from that of untreated women (Tables 3 and 4).

DISCUSSION

The proliferation rate of a cell population traditionally has been evaluated autoradiographically using [^{3}H]-thymidine and numerically by counting the number of visible mitoses. The first method evaluates those cells in the S phase and requires short-term incubation of breast tissue in culture medium.

Preliminary experiments showed that the high fat content of breast tissue from postmenopausal women considerably retarded [^{3}H]-thymidine penetration into the tissue (data not shown). This limitation precluded the use of [^{3}H]-thymidine incorporation as a reliable method. Counting mitotic figures identifies a small percentage of the cycling cells (only those in the short M phase), requires careful examination of thousands of epithelial cells, and provides reduced information of poorer quantity and quality with limited statistical value.

The proliferating cell nuclear antigen labeling index takes advantage of an endogenous marker that identifies all cells from the late G_1 through the G_2M phase. The interpretation of proliferating cell nuclear antigen immunoreactivity is well documented (20, 21). Computerized detection of cells that express proliferating cell nuclear antigen reduces subjectivity, improves reproducibility, and eases the workload. The proliferating cell nuclear antigen labeling index generates approximately 100 times more information than the mitotic index and therefore amplifies the validity of the cell-cycle analysis (15). Although flow cytometry determination of the cell cycle also is a reliable method (22), when applied to tissues, it does not discriminate between epithelial and stromal cells.

This study was designed as a double-blind, randomized trial in 40 postmenopausal women undergoing breast surgery for esthetic reasons or benign lesions. The daily percutaneous administration of estradiol, progesterone, combined estradiol and progesterone, or placebo for 14 days resulted in the expected changes in plasma and tissue concentrations of these steroids.

Estradiol significantly stimulated the proliferation of normal human breast epithelial cells in comparison with placebo. This proliferation index also was significantly higher than that associated with progesterone and combined estradiol and progesterone. Thus, progesterone administration dramatically limited the estradiol-induced proliferation of normal human breast epithelial cells.

Indeed, the progesterone and combined estradiol and progesterone groups showed comparably low levels of proliferation of galactophore cells that were, nonetheless, significantly higher than that of the placebo group. This low level of proliferating cell nuclear antigen–positive cells could reflect the capacity of progesterone to engage breast epithelial cells in the G_1 phase of the cell cycle.

The determination of objective thresholds of sensitivity demonstrates that the intensity of proliferating cell nuclear antigen labeling was significantly lower in the progesterone and combined estradiol and progesterone groups than in the estradiol group. The proliferating cell nuclear antigen–positive cells in the first two groups could represent those cells in the G_1 phase of the cell cycle that are not necessarily committed to undergo mitosis and proliferate because cells in the G_1 phase also may undergo apoptosis.

Our data strongly support the observations made by Barrat et al. (18) and by Chang et al. (15) that progesterone participates in the regulation of human breast epithelial cell proliferation. Our data also suggest that percutaneous progesterone may have a therapeutic value by antagonizing the estradiol-induced epithelial cell proliferation when applied for 14 days per month at substitutive doses. Isolated estrogen exposure (through either endogenous estrogen production or exogenous estrogen administration) may lead to sustained epithelial cell proliferation that can be counteracted by percutaneous progesterone.

Our results clearly differ from the findings reported by other investigators that indicated that optimum proliferation occurred during the luteal (progestogenic) phase of the menstrual cycle (2, 8–11) or in women who were taking an oral contraceptive containing a combination of estrogen and progestin or progestin alone (9). These data were interpreted as demonstrating a stimulatory influence of progesterone and progestins on breast epithelial cell proliferation. These conflicting data obtained using different techniques do not allow a definitive conclusion regarding whether progestins, either alone or in combination with estrogen, are breast mitogens.

Finally, it must be remembered that progesterone and synthetic progestins may induce different responses. Indeed, it is well known that the 19-nor-testosterone–derived progestins that are present in second- and third-generation oral contraceptives display an estrogenic potential in vitro and in vivo (23). They bind to the estradiol receptors and, in this way, stimulate human breast and endometrial cancer cell proliferation (23–25). The intrinsic estrogenicity of some progestogenic drugs therefore could explain, at least in part, these apparent controversies (26).

These discrepancies also may reflect the complexity of endocrine control of human breast cell proliferation. For example, in vitro studies may be misleading because they do not adequately reproduce interactions between epithelium and stroma. Some factors included in the culture medium may not be present in vivo, changing the microenvironment and thus the outcome. The conflicting in vivo results also might be the consequence of differences in the timing of hormone administration to our postmenopausal women in comparison with normal ovulatory cycles. Indeed, it is well known that steroid exposure leads to the synthesis and secretion of a variety of cytokines and growth factors by epithelial and stromal breast cells (27–29). The timing of endogenous steroid production and of exogenous steroid administration therefore may influence in various ways the synthesis of these autocrine and paracrine regulators of breast cell proliferation.

Our in vivo data indicate that, in contrast with indirect evidence from studies comparing [^{3}H]-thymidine incorporation with the day of the menstrual cycle, the direct administration of progesterone to postmenopausal women does not result in mitogenic activity of breast epithelium. Moreover, it is able to counteract the estrogen-induced proliferation of human mammary epithelial cells and, as a result, may protect against hyperplasia.

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