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Effects of hormone replacement therapy on the mammary gland of surgically postmenopausal cynomolgus macaques

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OBJECTIVE: Our purpose was to define the proliferative response and receptor status in the mammary glands of surgically postmenopausal macaques given hormone replacement therapy, equivalent for monkeys to that given women.

STUDY DESIGN: Surgically postmenopausal adult female cynomolgus macaques (*Macaca fascicularis*) were given either no treatment ($n = 26$), conjugated equine estrogens ($n = 22$), or combined therapy with conjugated equine estrogens and medroxyprogesterone acetate ($n = 21$). Drugs were administered in the diet, at doses equivalent on a caloric basis to 0.625 mg per woman per day for conjugated equine estrogens and 2.5 mg per woman per day for medroxyprogesterone acetate, for 30 months. Mammary gland proliferation was assessed subjectively and by morphometric and stereologic means. Estrogen receptor and progesterone receptor content and proliferation were studied by immunohistochemistry.

RESULTS: In this model combined therapy with conjugated equine estrogens and medroxyprogesterone acetate induced greater proliferation than did conjugated equine estrogens alone. The percentage of estrogen receptor -positive cells was decreased in the conjugated equine estrogens plus medroxyprogesterone acetate group. The percentage of progesterone receptor -positive cells was increased by treatment with conjugated equine estrogens alone.

CONCLUSION: These results indicate a proliferative response of mammary gland epithelium to therapy with

conjugated equine estrogens plus medroxyprogesterone acetate in postmenopausal macaques. The clinical implication of this finding may be a greater risk for development of breast neoplasms in women receiving combined hormone replacement therapy. (AM J OBSTET GYNECOL 1996;174:93-100.)

Key words:

Macaca fascicularis

hormone replacement therapy

mammary gland

steroid receptors

proliferation

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Postmenopausal estrogen replacement has been shown to have major beneficial effects in the prevention of coronary heart disease and osteoporosis. [1] Unfortunately, the public health benefits of hormone replacement therapy have not been realized, largely because of the fear of cancer. A recent report indicates that among women in the United States concern over the risk of breast cancer is the greatest deterrent for the use of hormone replacement therapy. [2] This concern has some basis in the results of recent epidemiologic studies that suggest an increased risk of breast cancer in long-term current users of hormone replacement therapy. [1] [3] [4] The mechanistic basis for this increased risk is unknown. The mitogenic effects of estrogens on both breast and endometrial tissue are well recognized, as are the beneficial effects of progestogens on endometrial cell proliferation and cancer risk. The great controversy concerns the action of progestogens on breast tissue, where the literature offers a number of conflicting results both in vitro [5] [6] [7] and in vivo. [8] [9] [10] [11] The assumption that breast and uterus are regulated similarly leads to the conclusion that the combined hormone replacement therapy designed to decrease the risk of endometrial cancer (i.e., estrogen plus a progestogen) is also appropriate for breast. A recent metaanalysis of studies including women treated with estrogen plus a pro-gestogen did not show a protective effect of progesto-gen use. [3]

Rhesus and cynomolgus macaques are similar to women in many aspects of reproductive physiologic and anatomic features. Macaques have a distinct menarche and menopause, at about the ages of 3 and 20 years, respectively. They have a 28-day menstrual cycle, with a

TABLE I -- Numbers of animals with atrophic or hyperplastic mammary glands by treatment

Treatment	Atrophy	Hyperplasia	Equivocal or not done
Control (n = 25)	23 (92%)	0	2 (8%)
CEE (n = 22)	11 (50%)	9 (41%)	2 (9%)
CEE plus MPA (n = 21)	2 (9%)	18 (86%)	1 (5%)

Numbers of animals with hyperplasia are significantly higher in conjugated equine estrogens plus medroxyprogesterone acetate group ($p = 0.0065$). *CEE*, Conjugated equine estrogens; *MPA*, medroxyprogesterone acetate.

hormonal profile similar to that of women. [12] Their endometrial responses to endogenous and exogenous hormones parallel those of women. [13] Mammary glands in these animals differ from the human breast grossly, but microscopically the mammary tissues of women and female macaques are quite similar. [14] Human and macaque mammary glands display the same cytokeratin types. [15] Mammary neoplasms are uncommon in macaques. [16] This is the first large study of the mammary responses of macaques to long-term hormone replacement therapy.

Methods

Animals.

The subjects of this study were 68 feral adult female cynomolgus monkeys (*Macaca fascicularis*) imported from Indonesia (Charles River Primates, Port Washington, N.Y.). The animals were part of an atherosclerosis-osteoporosis prevention trial, the results of which will be published elsewhere. They ranged in age from 5 to 13 years, as estimated from dentition, and were not pregnant. Animals were housed in social groups of four to eight monkeys each in a facility accredited by the American Association for Laboratory Animal Care. Experimental protocols were approved by the institutional Animal Care and Use Committee. Bilateral ovariectomies were done on all animals before the atherosclerosis induction period began.

Diets and drug dosing.

The hormones were administered twice daily in a moderately atherogenic diet (40% of kilocalories from fat, 0.2 mg of cholesterol per kilocalorie). Monkeys were fed approximately 120 calories/kg per day. For 8 months of the 30-month treatment period groups receiving conjugated equine estrogens were given 7.2 mug of conjugated equine estrogens (Premarin, Wyeth-Ayerst, Radnor, Pa.) per monkey per day. For the remaining 22 months the dose was approximately 166 mug per monkey per day to be equivalent to women receiving 0.625 mg per day. Throughout the 30-month treatment phase the group receiving conjugated equine estrogens plus medroxyprogesterone acetate were given approximately 650 mug per monkey per day of medroxyprogesterone acetate (Cycrin, Wyeth-Ayerst), to be equivalent to a woman's dose of 2.5 mg per day. Drug doses were computed as (Human dose)/(1800 kilocalories per woman per day) = Dose per calorie of diet.

Serum hormone measurements.

Before treatment measurements were made of estradiol and progesterone to confirm completeness of ovariectomy; estradiol and medroxyprogesterone acetate were measured during the treatment phase. Samples were taken 4 hours after feeding and dosing. Medroxyprogesterone acetate was measured by radioimmunoassay. Estradiol-17beta was measured by a modification of a commercial kit (Diagnostic Products, Los Angeles). All hormone measurements were carried out at the Comparative Endocrinology Laboratory of the Yerkes Regional Primate Center of Emory University (Atlanta) by Dr. Mark Wilson.

Tissue collection.

Mammary glands were collected at the end of the 30-month treatment phase, when all monkeys were killed and necropsies were performed. Samples were taken in the sagittal plane through the nipple and included a 2 to 3 cm segment of skin and gland. Tissues were fixed in 4% buffered paraformaldehyde for 24 hours and stored in 70% ethanol at 4° C. Tissues were then trimmed to 3 mm in thickness, embedded in paraffin, and sectioned at 5 μ m for immunostaining.

Histopathologic study.

Mammary gland slides were subjectively classified as atrophic, hyperplastic, or neither. The treatment group of each animal was obscured during the procedure to prevent observer bias. Hyperplasia, atypia, cystic lesions, and the presence of intraluminal protein or intraepithelial fat globules mimicking secretory activity were noted. Lesions were independently graded as none, mild, moderate, or severe.

Morphometry and stereologic study.

Mammary gland thickness was measured as greatest thickness perpendicular to the skin from histologic sections. Measurements were made with an ocular micrometer at a magnification of $\times 20$. An image analysis system (Bioquant, R and M Biometrics, Nashville, Tenn.) was used to measure mean nuclear area and nuclear roundness factor in 10 randomly selected cells each from alveoli, terminal ducts, and major ducts at a magnification of $\times 400$. Nuclear roundness factor is defined as $4\pi(\text{area})/\text{perimeter}^2$. Estimates of the relative proportions of tissue components in the mammary gland were made by point counting. [17] A 10×10 grid was superimposed on the section, and intercept points over features of interest were counted to determine the percentage of gland occupied by epithelium, connective tissue, and fat. Numbers of points intercepting each lobule were also recorded as a relative indicator of lobular size. For each section measured, 10 lobules were assessed, requiring an average of 4.6 microscopic fields at a magnification of $\times 20$.

Sex steroid receptors and proliferation.

Staining procedures were done on fixed, paraffin-embedded tissues.

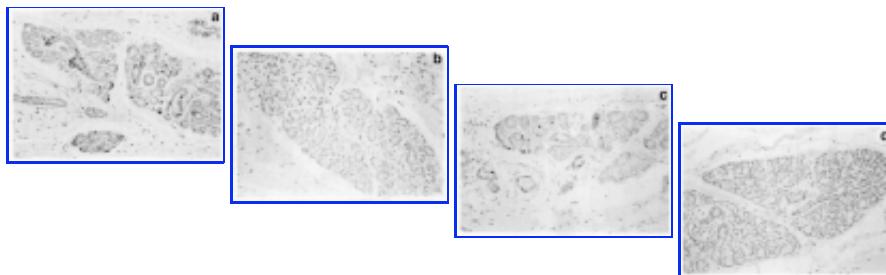


Figure 1. Typical estrogen receptor staining (a and b) and progesterone receptor staining (c and d) in control animals (a and c) and in animals from conjugated equine estrogens plus medroxyprogesterone acetate group (b and d).

Positively stained cells appear *black*. Loss of receptor staining is accompanied by increase in proportion of epithelial tissue.

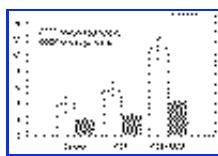


Figure 2. Point-counting measurements of percentage \pm SEM of epithelium relative to stroma and number of points counted per lobule in mammary gland of macaques. Both measures indicate glandular hyperplasia in conjugated equine estrogens (CEE) plus medroxyprogesterone acetate (MPA) group. For percent epithelium, the conjugated equine estrogens plus medroxyprogesterone acetate group differs from the conjugated equine estrogens group ($p < 0.05$) and from controls ($p < 0.0001$).

For points per lobule both conjugated equine estrogens -treated and conjugated equine estrogens plus medroxyprogesterone acetate -treated animals differed from controls ($p < 0.05$ and $p = 0.0007$, respectively) but did not differ from each other.

The basic staining procedure is an avidin-biotin-peroxidase method for antigen retrieval from paraffin-embedded tissue. [18] The estrogen receptor and progesterone receptor analyses were performed with reagents supplied by Dako Laboratories (Carpinteria, Calif.) and Immunotech Laboratories (Marseille, France), respectively. To assess proliferation, we used the newly introduced Ki-67 MIB-1 monoclonal antibody (Immunotech), which gives an immunostaining identical to Ki-67 antibody and which can be used on paraffin-embedded tissue sections. [18]

Quantification of immunohistochemical staining.

Immunostained cells were quantified by cell counting in

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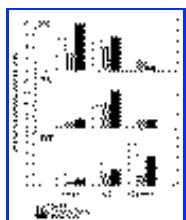


Figure 3. Immunostaining of mammary epithelial cells. MIB-1 labeling is increased in both treatment groups, most notably in conjugated equine estrogens (CEE) plus medroxyprogesterone acetate (MPA) group. Estrogen receptor (ER) immunostaining is decreased in conjugated equine estrogens (CEE) plus medroxyprogesterone acetate (MPA) group. Progesterone receptor (PR) immunostaining is significantly increased only in group given conjugated equine estrogens alone (Tables II and III).

sections by an observer blinded to treatments. Epithelial cells lining the alveoli and the terminal and major ducts were considered separately to assess regional differences. Cell nuclei were identified as unlabeled (0) or weakly (1), moderately (2), or intensely (3) labeled. At least 100 cells per slide were counted at three different sites for each combination of animal, tissue site, and stain type. Terminal ducts could not be identified in some cases.

Statistical methods.

Statistical analysis was performed with the Mann-Whitney *U* test with Bonferroni corrections for multiple comparisons, the Kruskal-Wallis test, chi² test, and Spearman's rank correlation test.

Results

Hormone measurements.

Plasma estradiol concentrations (mean \pm SEM) were 5.0 ± 0.7 , 167.1 ± 9.9 , and 160.9 ± 13.9 pg/ml for controls, conjugated equine estrogens, and conjugated equine estrogens plus medroxyprogesterone acetate groups, respectively ($p < 0.0001$ between control and treatment groups). The corresponding medroxyprogesterone acetate concentrations were 35.9 ± 6.1 , 24.7 ± 3.8 , and 116.2 ± 5.2 pg/ml ($p < 0.0001$ between untreated and medroxyprogesterone acetate - treated groups). Medroxyprogesterone acetate measurements for animals not given this drug were not significantly different from background.

Subjective evaluation of mammary morphologic features.

Mammary gland atrophy was seen in nearly all control animals. Animals given conjugated equine estrogens alone had lobular atrophy or hyperplasia with equal frequency. Eighty-six percent of animals given conjugated equine estrogens plus medroxyprogesterone acetate had mammary hyperplasia, defined as greater mammary gland development than that seen in a normally cycling premenopausal macaque ([Table 1](#)). Features similar to secretory differentiation were not related to treatment.

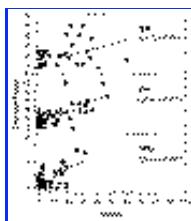


Figure 4. Regression plots of correlations between immunostaining in alveoli and terminal ducts. Correlations are highly significant. *ER*, Estrogen receptor; *PR*, progesterone receptor.

Morphometric and stereologic study.

Mammary gland thickness was significantly affected by treatment; the mean thickness (micrometers) \pm SD was 264 ± 153 for controls, 396 ± 211 for animals given conjugated equine estrogens, and 444 ± 249 for animals given conjugated equine estrogens plus medroxyprogesterone acetate. Controls differed from both treated groups at $p < 0.05$ and from the conjugated equine estrogens plus medroxyprogesterone acetate group at $p < 0.01$. The percentage of mammary gland occupied by glandular tissue was increased in both treated groups, most

markedly in animals given conjugated equine estrogens plus medroxyprogesterone acetate. Relative lobular size (expressed as points per lobule) was increased in animals given conjugated equine estrogens, and more so in animals receiving conjugated equine estrogens plus medroxyprogesterone acetate (Figs. 1 and 2).

Nuclear area was slightly increased in both hormonal treatment groups in the case of alveoli and terminal ducts. Nuclear roundness factor was slightly lower at all sites in the conjugated equine estrogens plus medroxyprogesterone acetate group. Nuclear changes did not reach statistical significance.

Immunostaining for estrogen and progesterone receptors and proliferating cells.

Table II illustrates the percentage of all receptor-positive cells for the different groups. The percentage of estrogen receptor -positive cells was decreased in both treatment groups, most markedly in the conjugated equine estrogens plus medroxyprogesterone acetate group (Fig. 3) . The percentage with positive staining for estrogen receptor and those intensely labeled () was higher in the control and conjugated equine estrogens groups than in the conjugated equine estrogens plus medroxyprogesterone acetate group. Significant differences were found between the total number of receptor-positive cells in the conjugated equine estrogens and conjugated equine estrogens plus medroxyprogesterone acetate groups for alveoli, terminal ducts, and major ducts. There were highly significant correlations between the percentages of estrogen receptor -positive cells of the alveoli and ducts ($r_s = 0.69, p < 0.0001$), alveoli and terminal ducts ($r_s = 0.70, p < 0.0001$), and between terminal ducts and ducts ($r_s = 0.64, p < 0.0001$) (Fig. 4) .

The percentage of progesterone receptor -positive cells was higher in the conjugated equine estrogens group than in both the control and conjugated equine estrogens

TABLE II -- Mean percentage of receptor-positive breast epithelial cells from cynomolgus macaques

	ER (mean, range)	PR (mean, range)
Alveoli		
Group A, control	13.7 (0-63) (n = 24)	3.2 (0-28) (n = 25)
Group B, CEE	12.9 (0-60) (n = 22)	19 (0-47)
Group C, CEE plus MPA	4.1 (0-28) (n = 19)	4.8 (0-32)
Significant differences	B vs C, $p = 0.014$	A vs B, $p = 0.0003$ A vs C, $p = 0.034$ B vs C, $p = 0.0006$
Terminal ducts		
Group A, control	7.1 (0-39) (n = 18)	5.0 (0-27) (n = 21)
Group B, CEE	10.1 (0-39) (n = 19)	20.7 (6-50)
Group C, CEE plus MPA	4.1 (0-31) (n = 18)	6.6 (0-25) (n = 19)
Significant differences	B vs C, $p = 0.035$	A vs B, $p = 0.0003$

Major ducts		<i>B</i> vs <i>C</i> , <i>p</i> = 0.0003
Group A, control	19.5 (0-76) (<i>n</i> = 26)	7.5 (0-43)
Group B, CEE	14.1 (0-52) (<i>n</i> = 22)	31.8 (0-56)
Group C, CEE plus MPA	1.7 (0-18) (<i>n</i> = 19)	7.7 (0-33)
Significant differences	<i>B</i> vs <i>C</i> , <i>p</i> = 0.003	<i>A</i> vs <i>B</i> , <i>p</i> = 0.0003
	<i>A</i> vs <i>C</i> , <i>p</i> = 0.007	<i>B</i> vs <i>C</i> , <i>p</i> = 0.0003

Number of evaluable specimens is indicated. *ER*, Estrogen receptor; *PR*, progesterone receptor; *CEE*, conjugated equine estrogens; *MPA*, medroxyprogesterone acetate.

TABLE III -- Mean percentage of cells with MIB-1 staining in breast epithelial cells of cynomolgus macaques

		Staining intensity	
		All stained cells (mean, range)	+++ only (mean, range)
Alveoli			
Group A, control (<i>n</i> = 25)	2.5 (0-19)	0.08 (0-2)	
Group B, CEE (<i>n</i> = 22)	5.4 (0-26)	0.14 (0-1)	
Group C, CEE plus MPA (<i>n</i> = 19)	8.0 (0-31)	0.84 (0-7)	
Significant differences	<i>A</i> vs <i>C</i> , <i>p</i> = 0.016	<i>A</i> vs <i>C</i> , <i>p</i> = 0.009	
Terminal ducts			
Group A, control (<i>n</i> = 20)	0.6 (0-3)	0	
Group B, CEE (<i>n</i> = 22)	2.1 (0-8)	0.04 (0-1)	
Group C, CEE plus MPA (<i>n</i> = 19)	1.9 (0-7)	0.13 (0-2)	
Significant differences	--	--	
Major ducts			
Group A, control (<i>n</i> = 26)	1.2 (0-10)	0	
Group B, CEE (<i>n</i> = 22)	3.0 (0-14)	0.32 (0-2)	
Group C, CEE plus MPA (<i>n</i> = 19)	5.5 (0-28)	0.84 (0-9)	
Significant differences	<i>A</i> vs <i>C</i> , <i>p</i> = 0.017	<i>A</i> vs <i>B</i> , <i>p</i> = 0.015	<i>A</i> vs <i>C</i> , <i>p</i> = 0.046

Number of evaluable specimens is indicated. *CEE*, Conjugated equine estrogens; *MPA*, medroxyprogesterone acetate.

plus medroxyprogesterone acetate groups in all three histologic sites ([Fig. 3](#)) . In all animals there were highly significant correlations between the percentages of progesterone receptor -positive cells of the alveoli and major ducts ($r_s = 0.76$, $p < 0.0001$), alveoli and terminal ducts ($r_s = 0.64$, $p < 0.0001$), and between terminal ducts and major ducts ($r_s = 0.64$, $p < 0.0001$) ([Fig. 4](#)) .

[Table III](#) shows the proportion of MIB-1 -positive cells for the different histologic cell types and treatments. The treated groups in general had a larger proportion of proliferating cells than controls, with the highest proportion in the conjugated equine estrogens plus medroxyprogesterone acetate group. Significantly higher values were seen in the conjugated equine estrogens plus medroxyprogesterone acetate group relative to untreated controls in alveoli and major ducts, and there were also significantly higher values for the conjugated equine estrogens group relative to

controls for intensely labeled cells in the major ducts. There was a strongly significant correlation between percentages of positive cells in alveoli and terminal ducts ($r_s = 0.40, p = 0.0025$, [Fig. 4](#)) and between alveoli and major ducts ($r_s = 0.32, p < 0.001$) but not between terminal ducts and major ducts.

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$p < 0.001$) but not between terminal ducts and major ducts.

Regarding serum concentrations of hormones, the following correlations had an r_s value of 0.25 to 0.5; all had a p value ≤ 0.05 . Higher serum concentrations of medroxyprogesterone acetate were positively correlated with MIB-1 labeling, lobular size, and percentage of the mammary gland section occupied by epithelial cells. Negative correlations were seen with estrogen receptor and progesterone receptor labeling. Higher serum concentrations of estradiol were positively correlated with MIB-1 labeling, progesterone receptor labeling, lobular size, and percentage of the mammary gland section occupied by epithelial cells. A negative correlation was seen between serum estradiol and estrogen receptor labeling.

When correlation testing was done for serum hormone concentrations within treatment groups, only medroxyprogesterone acetate concentrations were positively correlated with any immunostaining parameter (alveolar cells with positive MIB-1 staining, $r_s = 0.49, p = 0.035$, and strong alveolar staining, $r_s = 0.52, p = 0.024$).

Comment

Hormonal regulation of the normal breast and hormonal risk factors for the development of breast cancer remain a subject of controversy. In the normal menstrual cycle of women proliferation occurs primarily during the luteal phase of the cycle, indicating that breast does not respond to the same proliferative stimuli as the endometrium. [\[19\]](#) Human and nonhuman primate mammary glands have many similarities in anatomic features, hormonal regulation, [\[14\]](#) and cytokeratin immunophenotype [\[15\]](#) that are not shared by the commonly used laboratory rodents. We believe that the macaque model offers a unique opportunity for study of mammary gland regulation because it enables evaluation of the effects of long-term hormone replacement therapy on various locations in the breast of healthy subjects.

Morphometric and stereologic evaluation of tissues in this study clearly indicate a mammotropic effect of conjugated equine estrogens plus medroxyprogesterone acetate, which appears to exceed that of conjugated equine estrogens alone. This study shows a down-regulation of both estrogen and progesterone receptors in breast epithelium during combined treatment, similar to that of the endometrium. However, there was a significantly greater gland thickness and percentage of epithelial tissue in animals receiving combined therapy versus those receiving conjugated equine estrogens only. Also in contrast to the endometrium, there is a clear trend of increased proliferative activity of the breast epithelium on combined therapy. This is in line with studies that suggest an increased breast cancer risk associated with combined estrogen-progestin therapy. [\[10\]](#) It is, however, important to note that there is no statistical difference between Ki-67 (MIB-1) labeling in estrogen replacement therapy and combined hormone replacement therapy. The tendency for

increased proliferation in the combined therapy group was accompanied by decreased proportions of progesterone receptor -positive cells. Previously our group [20] and others [4] have found a sustained progesterone receptor level under the influence of progesterone during the luteal phase of the menstrual cycle. These findings indicate that estrogen receptors in breast are down-regulated by progesterone, as in uterus, [21] but that progesterone receptor positivity in breast does not change during the course of normal cycles. Progesterone is a well-known stimulator of lobulo-alveolar development. Apparently there are many differences between cyclic progesterone and continuous medroxyprogesterone acetate. We have recently shown that progesterone increases the intratissue formation of estrone from estrone sulfate, whereas norethisterone acetate in combined oral contraceptives does not because of differences in sulfatase activity induced by these two compounds. [22]

The basis of risk associated with hormonal therapies may lie in regulation of cell proliferation. Within populations of cells in vitro and in vivo, high rates of cellular proliferation increase the risk of transformation to the neoplastic phenotype. It is likely that this general phenomenon applies to the breast as well. [4] The murine monoclonal antibody Ki-67 reacts with a human deoxyribonucleic acid binding protein that is present in proliferating cells but absent in quiescent cells. A detailed cell-cycle analysis showed that the Ki-67 antigen is expressed in G₁, S, G₂, and mitosis (with maximum levels during G₂ and M phases) but not in G₀, and by use of this antibody an exact determination of the growth fraction of a given human cell population, regardless of whether it is normal or malignant, has been possible. [23]

The proliferation of breast cells in vitro has mostly been found to be stimulated by estrogens and inhibited by progestogens. However, in these experiments cultured epithelial cells are deprived of their normal complement of blood vessels, adipose tissue, stroma, and myoepithelial cells. These surrounding cells exert considerable paracrine and hormonal influence in vivo. Experiments with human tissue so far have been tritiated thymidine labeling and mitosis analyses on tissue sections from reduction mammoplasties or from "normal" breast tissue near a benign or malignant lesion. Most of these studies have demonstrated greater proliferation during the luteal phase. [4]

Clarke and Sutherland [24] postulated that progestogens could activate the cell cycle for one turn and that prolonged stimulation would turn it off. Our study seems to contradict this hypothesis because 2 years of prolonged continuous combined therapy significantly enhances breast cell proliferation. There is one report of a direct stimulatory effect of 19-nor steroids on estrogen receptor -positive breast cancer cells by the estrogen receptor. [25]

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The synthetic progestogen R 5020 stimulates insulin-mediated breast cancer cell proliferation by increasing insulin receptors and insulin receptor messenger ribonucleic acid content; this may be another mechanism of action on normal breast epithelial cells. [7] Further studies from our group will evaluate this hypothesis and also sex steroid receptor variation, breast cell proliferation, and growth factors of both cycling and oral contraceptive -treated healthy volunteers and cynomolgus monkeys.

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