Cyclic changes in the mammary gland of cynomolgus macaques

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Objective: To evaluate the influence of hormonal changes during the menstrual cycle on the mammary gland of female cynomolgus monkeys.

Design: Paired breast biopsy samples were obtained during the follicular and luteal phases of the cycle. Cycle characteristics were assessed by vaginal bleeding, serum hormones, vaginal cytology, and uterine ultrasound. The mammary gland was assessed by histology and immunohistochemistry for Ki67, estrogen receptors (ER) alpha and beta, progesterone receptors (PR), and cleaved caspase 3 (CPP32).

Setting: Nonhuman primate study in an academic research environment.

Animal(s): Fifty-two adult, female, feral cynomolgus macaques (Macaca fascicularis), aged 8 to 20 years, obtained from the breeding colony of the Institut Pertanian Bogor (Bogor, Indonesia).

Main Outcome Measure(s): Breast histomorphometry, immunohistochemical detection of Ki67, ERs, PR, and CPP32 in breast epithelial cells, and correlation with serum estradiol and progesterone.

Result(s): Serum hormones, vaginal cytology and bleeding patterns were indicative of cycle stage. For lobules, Ki67 expression was higher in the follicular than in the luteal phase. In ducts, Ki67 expression was higher in the luteal than in the follicular phase. Estrogen receptors did not change across the cycle, but ER beta was more abundant. Ductal PR decreased in the luteal phase. Lobular CPP32 was higher during the luteal phase. Correlations of serum estradiol to outcomes varied by cycle stage.

Conclusion(s): These data indicate important regulatory differences in the balance of proliferation and apoptosis in epithelial subpopulations within the breast across the menstrual cycle, indicating different regulatory roles for ER alpha and beta. (Fertil Steril 2004;82(Suppl 3):1160–1170. ©2004 by American Society for Reproductive Medicine.)

Key Words: Breast, menstrual cycle, proliferation, apoptosis, monkeys, estrogen receptor, progesterone receptor

There is considerable uncertainty as to how, or even which, ovarian steroids influence premenopausal and postmenopausal breast epithelial cell proliferation and subsequently breast cancer. Estrogen is generally accepted as a promoter of the proliferation of the breast epithelial cell. The role of progesterone on breast epithelium cell proliferation remains controversial, primarily because of the difficulty of obtaining normal breast tissue and the relative paucity of models of progesterone action in the normal breast. Breast cancer cells have been used extensively as models to examine progesterone effects. However, the limitation of studying progesterone regulative mechanisms in malignant cells is the difficulty in extrapolating results to the normal breast (1). In vitro studies of the involvement of progesterone in normal breast epithelial proliferation have produced inconsistent results. Progesterone has been found to increase DNA synthesis in normal mammary epithelium in organ culture (2). However, progesterone either decreases or has no effect on the proliferation of normal breast epithelium explanted into nude mice (3, 4). The addition of estrogen and the progestin R5020 to cultured normal breast epithelial cells had opposing effects, estrogen increasing and progesterone decreasing proliferation (5).

Similarly, there are controversial results from in vivo studies. There is general agreement that an increase in DNA synthesis is seen in the late
luteal phase of the natural cycle (6–11). The increase in DNA synthesis is consistent with the observation of a cyclical increase in the number of epithelial mitoses, which peaks toward the end of the luteal phase and is followed by an increase in apoptotic activity (12, 13). However, these findings do not prove that progesterone is the major steroid mitogen. Indeed, in studies of human breast tissue implanted into athymic nude mice, no effect of progesterone could be demonstrated when it was used alone or in combination with estradiol (14). In respect to postmenopausal breast epithelial cell proliferation, data from clinical trials of hormone therapy (HT) support the hypothesis of a proliferative effect of progesterone. Meta-analyses and reanalyses of epidemiologic data have revealed that current use of HT slightly increases the relative risk of breast cancer (15, 16). Combined estrogen/progestin regimens have been shown to be associated with a statistically significant higher risk of breast cancer than treatment with estrogen alone (17–19). The effect of oral-contraceptive use is less clear, although there seems to be a slight increase in breast cancer risk in long-term users, and there is a possible increase in risk associated with progestogen-containing oral-contraceptive use before the first full-term pregnancy (20, 21). These disparate observations increase the uncertainty regarding the role of progestins, thus adding to the urgency of understanding how breast tissue is regulated.

The effects of estrogen and progesterone are mostly mediated via sex steroid receptors. In the mammary gland, estrogen receptor (ER) alpha and beta are present in the epithelial cells of alveoli and ducts as well as in stromal cells (22, 23). Previous studies have shown that ER-alpha expression in breast epithelial cells is down-regulated in the luteal phase, whereas progesterone receptor (PR) expression remains at a high level throughout the cycle (24–26). Recently, ER-beta expression has been shown in the breast (26–28). There is some evidence that its expression relative to the menstrual cycle remains constant (26). However, for obvious reasons, it is difficult to study cyclic variations in the normal breast in women in vivo. Studies from autopsy material or material from reduction mammoplasties or near fibroadenoma tissue only permit the study of tissue from a single occasion, given the difficulty of performing repeated examinations of the same woman (5, 10, 13, 26).

In this study we have chosen a cynomolgus macaque (Macaca fascicularis) model to evaluate the influence of hormonal changes during the menstrual cycle on the mammary gland. Cynomolgus macaques are similar to women in menstrual cyclicity and gonadal hormone profile. Further, as among women, their reproductive activity is not strongly seasonal. Using this model, breast and reproductive tract effects for conventional hormone replacement therapy have been evaluated in monkeys in several studies and found to be analogous to those seen in women (29–34). Other investigators have used macaques for a wide variety of studies of reproductive physiology (35–43), clearly demonstrating the utility of the model. We and others have recently reported that the prevalence of preneoplastic and cancerous lesions in macaques is higher than previously reported, may be similar to that seen in women (44), and these neoplasms express ER-alpha and cell-surface erbB-2 (45).

The aim of the prospective study was to examine the effects of endogenous progesterone and estrogen on markers of breast cancer risk and to evaluate indicators of cycle stage in the cynomolgus macaque model. As normal breast proliferative activity undergoes cyclic changes, an extended knowledge of normal breast function should improve the understanding of tumor development.

**MATERIALS AND METHODS**

**Animals and Treatments**

Fifty-two adult female feral cynomolgus macaques ranging from 8 to 20 years of age (mean: 16 ± 4 years) were obtained from the breeding colony of the Institut Pertanian Bogor (Bogor, Indonesia). The animals were considered multiparous because over 90% of the adult females in the originating colony had given birth at least once since sexual maturity, which occurs at 4 years of age in this species.

The animals were housed in social groups of six animals each. Each animal was clinically evaluated and determined to be in good health. Body weights were measured monthly during the course of the study. The animals were fed a diet formulated to model typical North American consumption, with a wheat flour base and deriving 43% of calories from fat (mostly saturated), 18% from protein, and 39% from carbohydrates. The diet is similar in composition to those used previously (46). This diet does not adversely affect reproductive function in females and was used to avoid the well-known phytoestrogenic effects of commercial laboratory animal diets (47). Food was provided once daily, in 5% excess of the amount consumed by each group. All procedures involving animals were conducted in accordance with state and federal regulations and were approved by the Institutional Animal Care and Use Committee of the Wake Forest University School of Medicine.

**Menstrual Cycle Characteristics and Biopsy Designation**

Menstrual cycle characteristics were evaluated for 6 consecutive months. As part of this process, animals were trained to present their hindquarters for daily vaginal swabbing. The onset of menses was considered day 1 of the cycle. On the basis of detailed, daily swabbing records, luteal and follicular phase breast tissue biopsies were scheduled beginning in month 4 of evaluation and continuing through month 6.

**Serum Hormone Concentrations**

Blood samples for the determination of total serum estradiol and progesterone concentrations were obtained from
each animal on the biopsy dates before surgery and following an overnight fast. Hence, each animal was represented by both a luteal and a follicular phase hormone sample. Serum concentrations of progesterone were measured by radioimmunoassay (RIA) using a commercially prepared kit (Diagnostic Products Corp., Los Angeles, CA). With 100 μL serum, the assay has a sensitivity (80% bound to free ratio) of 0.3 ng/mL. Intra-assay and interassay coefficients of variation (CV) were 3.1% and 8.7%, respectively. Serum estradiol was measured by a direct RIA using a modification of a commercially available kit (Diagnostic Products Corp.). Rather than using human serum as the diluent, serum from gonadectomized monkeys was treated with charcoal to remove any residual steroids and was subsequently used as the diluent in the assay. Using 200 μL serum, the assay sensitivity ranged from 7–10 pg/mL. Interassay and intraassay CVs averaged 10.5% and 3.1%, respectively (48).

Vaginal Cytology and Ultrasound Examination

Vaginal cytologic evaluation and ultrasound examinations of the uterus were done each time before performing the breast biopsy. Vaginal cytologic samples were taken from the vaginal mucosa and stained using the Papanicolaou method, as we have described previously elsewhere (29). Numbers of parabasal, intermediate, and superficial/keratinized cells were counted, and the maturation index (numbers of cells of each type), karyopyknotic index (percentage of cells of each type), and maturation value (MV) were analyzed. The equation used to generate maturation value was that of Meisels (49); that is, MV = 0.2 (Number of parabasal cells) + 0.6 (Number of intermediate cells) + (Number of superficial cells).

Ultrasound examinations of the uterus were performed using methods described by Foster et al. (50). Briefly, ultrasound examinations were performed using a SonoSite Ultrasound System 180 with a 5.0 MHz linear transducer (SonoSite Inc., Bothell, WA). The transducer was placed on the lower abdomen on the midline, the uterus was viewed in a transverse plane, and endometrial thickness was measured by omnidirectional calipers on a static ultrasound image. The thickness reported is the double endometrial thickness from one endometrial cavity to the other. Analysis of the endometrium and uterus itself was performed by consensus.

Breast Tissue Biopsy

Luteal and follicular phase breast samples (approximately 200 mg each) were taken from each of the 52 animals. Animals were trained to enter a small transport cage, where they were anesthetized by intramuscular injection of ketamine (10 mg/kg) and butorphanol (0.025 mg/kg). The procedure was done on a heated table; nasal insufflation of oxygen was given, and monkeys were monitored continuously during the brief surgery for heart rate and oxygenation by pulse oximetry. The right breast was chosen for the first, and the left breast for the second biopsy. Breast biopsies were performed using standard aseptic surgical technique. A small (1 to 2 cm) incision was made in the skin of the upper outer quadrant of the breast, followed by removal of a 200- to 300-ng/mL sample of breast tissue. After removal tissue was immediately fixed in 4% paraformaldehyde at 4°C. The skin was closed using subcuticular suture followed by surface application of surgical adhesive, and the site was tattooed to prevent later sampling at the same site. Animals were monitored closely during postoperative recovery and treated with analgesics for any signs of discomfort. After 24 hours, tissues were transferred into 70% ethanol at 4°C, then trimmed to 5 mm in thickness, embedded in paraffin, and sectioned at 5 microns for hematoxylin and eosin (H&E) staining, or placed onto charged slides (Probe-on Plus, Fisher Scientific, Pittsburgh, PA) for immunohistochemistry.

Histology

End points included histopathologic and histomorphometric characterization of the mammary gland. Extralobular ducts, intralobular ducts, and alveoli were evaluated separately. Lobular enlargement was expressed relative to the most atrophic animals among the examined cohort, and was graded as not present, minimal, mild, moderate, or marked; the predominant lobular type was also assessed using the criteria of Russo et al. (51). All slides were reviewed by a primary board-certified veterinary pathologist (JMC) and a second DVM (CEW). Differences of opinion were resolved by consensus.

Histomorphometry

The histomorphometric evaluation was a relatively gross measure intended to provide an overview of epithelial density within the breast. Histomorphometric measurements included the percentage of the tissue examined that was occupied by glandular epithelial tissue, expressed as a percentage of the total area examined. All morphometric measurements were done on H&E-stained slides, using a video image analysis system consisting of a Hitachi VK-C370 camera, a Power Macintosh G3 (Carpenteria, CA), a video capture board, Scion LG-3 (Scion, Inc., Frederick, MD), and public domain software (NIH Image v1.60, available via the Internet at http://rsb.info.nih.gov/nih-image/). For each animal, three microscopic fields were randomly selected and examined at a magnification of ×20, and mammary glandular structures were traced manually for measurement of areas as per our previously published methods (32).

Immunohistochemistry

Immunostaining was performed using commercially available primary antibodies for the proliferation marker Ki67 (Ki67-Mib 1; Dako, Carpinteria, CA) as well as PR (NCL-PGR; Novocastra, Newcastle-upon-Tyne, United Kingdom), ER-alpha (NCL-ER-6F11; Novocastra), ER-beta (MCA 1974, clone PPG5/10; Serotec, Raleigh, NC), and the apoptosis marker cleaved caspase 3 (CPP32, clone ASP
175]; Cell Signaling Technology, Beverly, MA). Antigen retrieval was performed with citrate buffer (pH 6.1) (52, 53), with biotinylated rabbit anti-mouse Fc antibody as a linking reagent, and alkaline-phosphatase conjugated streptavidin as the label. The chromogen used was Vector Red; immunostaining components were obtained as a kit (Vector Laboratories, Burlingame, CA).

Immunohistochemical staining for Ki67, PR, ER-alpha and ER-beta, and CPP32 was quantified by computer-assisted counting technique, using a grid filter to select cells for counting, using our previously described modification (31, 32) of the method of cell selection described by Lindholm et al. (54). This method allows the user to randomly and objectively subsample large areas of the tissue encompassing several thousand cells without exerting selection bias toward positive cells. Cells were counted in large (extralobular) ducts and in terminal ductal-lobular units (TDLU) consisting of alveoli and intralobular small ducts. Numbers of cells were expressed as a percentage of the total number examined (100 cells sampled from each tissue site within each breast section). Positively labeled cells were graded as weakly, moderately, or strongly labeled. Interobserver and intraobserver correlations using this method are high (0.90, P<0.05).

Statistical Methods

Analyses were done using JMP Statistical Discovery Software (Version 3.2.2; SAS Institute, Inc., Cary, NC). One-way analysis of variance (ANOVA) was used to test the mean for differences among all groups; further pairwise t-tests were used to identify differences between cycle phases if the overall ANOVA indicated that statistically significant differences were present. An alpha level of 0.05 was used for all statistical tests.

RESULTS

Body Weights

Before the study, there was a statistically significant gain in body weight during the 3-month importation quarantine, from a mean of 2.65 kg (± standard deviation [SD] of 0.33) on arrival to 3.02 ± 0.40 kg at the end of quarantine (P<0.05). There were no statistically significant changes in body weight during the course of the study; at the end of the study, body weights averaged 2.99 ± 0.43 kg.

Confirmation of Cycle Phase

Vaginal Swabbing

Daily swabbing before and after all biopsies indicated that, based on bleeding patterns, follicular biopsy samples were obtained on day 11 ± 1.5 and luteal samples on day 21 ± 1.6. Vaginal cytologic assessments indicated that the maturation value (MV) was statistically significantly higher in the follicular than in the luteal phase of the menstrual cycle (MV mean difference 1.46 ± 0.7; P<0.05).

Endocrinology

Estradiol showed a statistically significant elevation in the follicular phase when compared with the luteal phase (62.3 ± 8.46 pg/mL vs. 39.07 ± 4.61 pg/mL; P<0.05). In contrast, progesterone showed a statistically significant increase during the luteal phase (1.05 ± 0.1 ng/mL vs. 3.26 ± 0.31 ng/mL; P<0.05).

Ultrasoundography

Double endometrial thickness was 0.51 ± 0.02 cm in the follicular phase and 0.51 ± 0.02 cm in the luteal phase. Endometrial area was 0.41 ± 0.02 cm² in the follicular phase and 0.44 ± 0.02 cm² in the luteal phase. Uterus area was 1.90 ± 0.08 cm² in the follicular phase and 2.02 ± 0.08 cm² in the luteal phase. For any of the parameters, a statistically significant difference could not be found between the follicular and luteal phases (P=0.34).

Histopathologic Findings

No neoplasms were seen in any group, and the degree of lobular development was within normal limits for all animals in the study. Most lobules corresponded to type 2 in the human classification of Russo et al. (51), with an approximate mean of 35 alveoli per lobule. Minimal to mild increases in lobular size were seen in 80.8% of animals in the follicular phase versus 88.5% in the luteal phase; this difference was not statistically significant. A single animal had focal ductal hyperplasia, found in the luteal phase.

Change of Markers across the Cycle

Mammary Epithelial Tissue

The percentage of the mammary gland occupied by epithelial tissue was slightly, but not statistically significantly, increased in the luteal phase of the menstrual cycle compared with the follicular phase. On average, epithelial tissue comprised 8% to 9% of the biopsy samples.

Proliferation Marker Ki67

Expression of the proliferation marker Ki67 in breast lobuloalveolar cells was statistically significantly higher in the follicular than in the luteal phase of the menstrual cycle (Ki67 alveoli: follicular, 31.6 ± 2.5%; luteal, 25.5 ± 2.2%; P<0.05). This difference was not observed in ductal cells. Looking at strongly labeled cells only, the expression of the proliferation marker Ki67 in lobuloalveolar cells did not differ between follicular and luteal phase (P=0.19). However, in ductal cells, strong Ki67 expression was statistically significantly elevated during the luteal phase (Ki67 ductal cells: follicular, 2.9 ± 0.4%; luteal, 4.3 ± 0.7%; P<0.05). Stroma was not labeled (Fig. 1).

Progesterone Receptor

Figure 2 shows PR expression, as found in 89% of breast samples. On average, PR labeling was higher in lobuloalveolar cells than in ductal cells, without reaching statistical
In ductal cells, PR expression was statistically significantly higher in the follicular than in the luteal phase (PR ductal cells: follicular, 9.02 ± 1.2%; luteal, 5.05 ± 1.2%; P < 0.05).

In lobuloalveolar cells, the amount of PR-positive cells tended to be higher during the follicular phase, but the difference was statistically insignificant (PR alveoli: follicular, 10.87 ± 1.4%; luteal, 8.2 ± 1.5%; P = 0.072). No labeling in stromal tissue was detected.

**Apoptosis Marker CPP32**

Apoptosis data are shown in Figure 3. Expression of the apoptosis marker CPP32 in breast lobuloalveolar cells was statistically significantly higher in the luteal than in the follicular phase of the menstrual cycle (CPP32: follicular, 3.5 ± 0.8%; luteal, 5.5 ± 0.99%; P < 0.05). This difference could not be found in ductal cells. In general, CPP32 labeling was higher in lobuloalveolar than in ductal cells. In follicular as well as in luteal phase, CPP32 expression was only seen in about 1% of stromal cells.

**Estrogen Receptor Alpha and Beta**

Expression of ER-alpha and ER-beta was found in 96% and 100% of breast samples, respectively. On average, statistically significantly more ER-alpha and beta labeling was found in breast lobuloalveolar than in ductal cells, in both follicular and luteal phases (P < 0.0001). For ER-alpha, the mean percentage of positively labeled cells was 44% in lobuloalveolar and 14% in ductal cells. The corresponding data for ER-beta were 73% and 56%, respectively. The amount of cells positive for ER-beta cells was statistically significantly higher than of cells positive for ER-alpha, independent of cycle stage and breast tissue type (Fig. 4).

For both ERs, no statistically significant differences between follicular and luteal phase could be found. The ER-alpha/ER-beta-ratio remained unchanged across the cycle in both lobuloalveolar and ductal cells.

**Markers and Their Relation to Serum Hormones within Cycle Phases**

Statistically significant correlations between serum hormones and tissue outcomes are summarized in Table 1. During the follicular phase of the menstrual cycle in lobuloalveolar tissue as well as in ducts, ER-alpha expression was negatively correlated with serum estradiol concentrations (r = −0.46, P < 0.001; r = −0.27, P = 0.053, respectively). In contrast, ER-beta expression was positively correlated with increasing serum estradiol concentrations in
lobuloalveolar tissue ($r = 0.3$, $P<.05$). This finding was supported by the ER-alpha/ER-beta ratio being inversely correlated with serum estradiol concentrations in alveoli ($r = -0.47$, $P<.001$) and ducts ($r = -0.29$, $P<.05$). During the luteal phase of the menstrual cycle, serum estradiol concentration was correlated with labeling for Ki67 in ducts ($r = 0.3$, $P<.05$) and PR in alveoli ($r = 0.38$, $P<.01$).

Associations between dependent variables were also seen. The percentage of the breast tissue occupied by epithelium was negatively associated with ER and PR ($r = -0.031$, $P<0.05$ for ER; $r = -0.37$, $P<0.01$ for PR). Estrogen receptor alpha was also negatively correlated with Ki67 ($r = -0.40$, $P<0.01$), but this was not true for ER-beta, which was positively correlated in the luteal phase ($r = 0.27$, $P<0.05$). Estrogen receptor alpha was positively correlated with CPP32 ($r = 0.40$, $P<0.01$), as was the ER-alpha/beta ratio, whereas ER-beta was negatively correlated with CPP32 ($r = -0.36$, $P<0.01$). In both sites and both cycle phases, PR was strongly associated with CPP32 expression ($r = 0.53$, $P<0.0001$), and PR was also positively correlated with Ki67 expression ($r = 0.33$, $P<0.05$). Also, Ki67 and CPP32 were correlated to each other ($r = 0.32$, $P<.05$).

**DISCUSSION**

This is the first study to report changes in the macaques’ mammary gland during the menstrual cycle. Major findings include the following. For ducts, Ki67 expression was statistically significantly higher in the luteal than in the follicular phase, whereas the opposite was found in alveoli. Estrogen receptor expression remained stable across the cycle with ER-beta expression being higher than ER-alpha expression. Progesterone receptor expression decreased from the follicular to luteal phase, whereas CPP32 expression was higher during the luteal phase.

Our data also indicate that the simplest and least invasive method for determining cycle stage in the cynomolgus model is the assessment of vaginal bleeding and serum hormone concentrations. Luteal-phase serum progesterone was slightly lower in this study than in previous work from our group and may indicate a degree of suppressed ovarian cyclicity in these recently imported animals.

Ultrasonographic measurement of the endometrium was not a useful tool for cycle stage dating. This may be due to the late stage of the follicular phase when breast biopsies and ultrasonography were performed: endometrium diameters are not likely to differ significantly at day 11 and 21 of the cycle. Vaginal keratinization differed in a statistically significant fashion between the two cycle phases, but the low numerical difference between phases indicates that this measure is of limited usefulness.
In this study, the amount of mammary epithelial area remained unchanged across the cycle, whereas expression of the proliferation marker Ki67 was statistically significantly higher in the luteal phase for ducts. This finding is of importance because the site of origin of most breast cancers is the terminal ductal-lobular unit of the breast. Our results are supported by Clarke et al. (55), who showed a significant increase of epithelial proliferation in normal human breast tissue xenografted into athymic nude mice when treated with estradiol at luteal phase serum concentrations, alone or in combination with progesterone. Furthermore, several in vivo studies in humans reported higher proliferation indices during the luteal phase (6–8, 12, 13, 56–59). In general, the number of proliferating cells in breast tissue reported by these investigators was much lower than our finding. This might be due to the use of different proliferation markers like mitotic index (12, 13, 57, 58), proliferating cell nuclear antigen (PCNA) labeling (58), or thymidine labeling index (6–8), but even in studies also using Ki67 labeling as a proliferation marker, the percentages of Ki67-positive cells were lower (59). However, the investigators did not differentiate between lobuloalveolar and ductal breast tissue: the amount of Ki67-labeling cells reported in previous studies corresponds to our findings in ducts. Furthermore, none of the studies except one (59) investigating the influence of the menstrual cycle on breast tissue were performed on breast biopsies paired for follicular and luteal phase in the same woman.

There also might be a difference between the human and nonhuman primate mammary gland with respect to the total amount of Ki67-positive cells. But despite this difference in means, in some premenopausal women the proportion of proliferating cells has been reported to be as high as 50% (60); thus, the model described here may be more applicable to the subset of women with more breast proliferation. Our findings disagree with those of Chang et al. (58), which showed that progesterone lowers and estradiol increases breast proliferation. However, in that study breast proliferation markers were not determined during the spontaneous menstrual cycle but rather after topical application of estrogen and/or progestin to the breast during follicular phase, presumably achieving tissue hormone concentrations much higher than physiologic levels.

Estrogen is known to stimulate not only proliferation but also PR synthesis in the epithelial component of normal breast. This effect on PR is mediated by ER; therefore, the presence of PR is indicative of a functional ER. In our study, PR expression was found in approximately 80% of breast biopsies across the cycle, which has been supported by studies in women (24, 25). The amount of PR-positive cells decreased from follicular to luteal phase, which reached a

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**FIGURE 3**

Percentages of cells within the mammary gland epithelium labeling positively for CPP32 by immunohistochemistry. Red = lobuloalveolar epithelial cells; Blue = ductal epithelial cells. Staining in lobuloalveolar cells was greater in the luteal phase (* = P<0.05).

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statistically significant difference only for ducts. In contrast, some investigators have reported a stable amount of PR expression across the cycle (24, 26, 61); however, in those studies PR was not always quantified separately in alveoli and ducts. Furthermore, different techniques of breast tissue collection such as biopsy or fine-needle aspiration, as well as different labeling methods, possibly contributed to different findings.

Because tissue homeostasis is the result of proliferation, differentiation, and apoptosis, we also performed an immunohistochemical investigation of apoptosis by using an antibody specific for the active form of CPP32. Although the activation of the CPP32 cascade is an early event of apoptosis, once it has begun it is irreversible (62). Furthermore, the method is more sensitive and specific than morphologic evaluation, DNA fragmentation assessment, or terminal uridine nucleotide end labeling techniques. In alveoli, CPP32 expression was statistically significantly higher in the luteal than in the follicular phase. Both ducts and stroma expressed CPP32 in approximately 1% of cells across the cycle. Our finding is supported by studies based on morphologic identification of apoptosis, showing an apoptotic peak at day 28 of the cycle (56). Sabourin et al. (63) demonstrated a cyclic variation in bcl-2 expression, an antiapoptotic protein, in normal breast tissue, with a maximal expression at the end of the follicular phase and a progressive decrease during the luteal phase. In contrast, Potten et al. (57) did not report a variation of apoptosis in breast tissue across the cycle. Another investigation (64) of CPP32 expression in benign breast tissue reported that 8% of cells were positively labeled.

In our study, ER-alpha expression was present in nearly all breast samples (96%) examined, with the percentage of positive cells being higher in alveoli (44%) than in ducts (14%) across the cycle. This rate of expression and percentage of positive cells is higher than reported in women: Battersby et al. (24) reported the ER-alpha expression was found in 60% of women in the follicular phase; Soderqvist et al. (25) reported ER-alpha expression in 68% of women in the follicular phase and 32% in luteal phase. In those women expressing ER, the percentages of cells positive for ER-alpha ranged from 0.76% (65) to 10% to 25% (25–28, 55, 65–67).

A small number of studies have investigated the influence of hormonal changes during the menstrual cycle on the expression of ER in the breast. In contrast to previous studies in women reporting higher ER-alpha expression levels in the follicular than the luteal phase (24–26, 61), we found that ER-alpha expression remained stable through the cycle in both lobuloalveolar and ductal tissue. We found similar expression patterns for ER-beta, which was in line with the findings of other investigators focusing on ER-beta expres-
The observed lower expression of ER in previous studies may relate to how “normal” breast tissue was defined because some of the studies were performed in tissue adjacent to benign or malign tumors (28, 67, 68) or in mammoplasties (26, 65, 69), which may not be entirely normal. Furthermore, ER-alpha expression in many studies has yet to be correlated with age, treatment, or cycle stage (55, 61, 65, 67–71), although it is known from studies in rodents that ER-alpha expression varies according to the reproductive phase (27) and is influenced by hormonal treatment, as was shown in women and nonhuman primates given HT or oral contraceptives (11, 24, 30). Different methods of immunohistochemical staining and counting of positively labeled cells also may contribute to different results.

Only a few studies have investigated the expression of ER-beta in the normal breast. In our study, ER-beta expression was found in all breast samples, with approximately 73% of cells in alveoli and 56% of cells in ducts being positively stained. These findings are supported by studies in rodents, in which approximately 60% to 70% of epithelial cells expressed ER-beta at all stages of breast development (27). In humans, Roger et al. (28) reported 80% of epithelial cells were positive for ER-beta, whereas Shaw et al. (26) only found 25% of epithelial, myoepithelial, and stromal cells to be positively labeled.

Correlation analysis in this study revealed that ER-alpha and beta react differently in response to estradiol, and appear to play different regulatory roles in the breast. For example, estradiol was associated with lower ER-alpha expression in the follicular phase of the cycle but was strongly associated with higher ER-beta expression. With respect to other hormone-dependent measures, ER-alpha was negatively correlated with Ki67 and mammary epithelial area, whereas ER-beta was positively correlated in other comparisons. Estrogen receptor alpha and increased ER-alpha/beta ratio were positively correlated with CPP32 expression in ducts, implying that differential expression of the two ER forms is important in regulation of breast cell survival. Estrogen receptors and PR were both negatively correlated with epithelial area, indicating that more developed, larger lobuloalveolar units had less sex-steroid receptor expression. Progesterone receptors were strongly associated with CPP32 expression in both cycle stages and both cellular sites examined, and also were correlated with Ki67 expression; Ki67 and CPP32 expression were positively associated. These findings most likely represent components of an estrogen-dependent response, because PR and cell proliferation are well-known estrogenic responses, and increases in apoptosis usually accompany increases in cell proliferation.

In conclusion, expression of ER-alpha and ER-beta remains unchanged across the cycle, with ER-beta expression being statistically significantly higher than ER-alpha expression independent of cycle stage and cellular site. However, ER-alpha and beta have different associations with serum estradiol and breast proliferative responses, and thus are likely to have different regulatory roles in the breast. In particular, the ER-alpha/beta ratio may be important. With respect to cellular proliferative changes across the cycle, ductal but not lobuloalveolar proliferation was higher during the luteal phase. Apoptosis was statistically significantly higher in the luteal phase, although this was only true for

![Figure 5](image_url)

alveoli. In contrast, in ducts, apoptosis per se was low and did not change in a statistically significant manner across the cycle. Thus, in alveolar tissue during the luteal phase, apoptotic events may outweigh proliferation events whereas in ducts the opposite may be true. The observed different proliferation patterns for alveoli and ducts resulting from cycle-specific hormonal variations are important because the site of origin of most breast cancers is the terminal duct–lobular unit of the breast.

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References


Table 1

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<tr>
<th>Cycle stage</th>
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<td>52</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ducts</td>
<td>Estradiol</td>
<td>ER-alpha</td>
<td>-0.27</td>
<td>52</td>
<td>0.0526</td>
<td></td>
</tr>
</tbody>
</table>

Note: ER = estrogen receptor; PR = progesterone receptor; CPP32 = cleaved caspase 3.


