

Proliferation of breast epithelial cells in healthy women during the menstrual cycle

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OBJECTIVE: Our objective was to assess proliferation in normal breast epithelial cells from healthy women during the follicular and luteal phases of the menstrual cycle.

STUDY DESIGN: We analyzed the proliferation marker Ki-67/MIB-1 by immunocytochemical methods in breast epithelial cells procured through fine needle aspiration biopsy from 47 healthy volunteers. Differences were assessed by Wilcoxon rank sum tests, and correlations were determined by Spearman's rank correlation coefficient.

RESULTS: The proportion of Ki-67/MIB-1-positive cells was higher in the luteal phase (2.04%) than in the follicular phase (1.66%). The values in women aged <35 years were 2.29% and 1.13%, respectively ($p = 0.003$). In ovulating women with two aspirates during the same menstrual cycle the percentage of proliferating cells increased from the follicular phase (1.3%) to the luteal phase (2.4%) ($p < 0.04$). Proliferation was positively correlated with serum progesterone levels the day of aspiration ($r = 0.34$, $p < 0.05$).

CONCLUSION: The fine needle aspiration biopsy technique is a valuable tool for in vivo studies of cell proliferation in the normal breast. Data clearly suggest a proliferative action of progesterone. (Am J Obstet Gynecol 1997;176:123-8.)

Key words: Breast epithelial cells, proliferation, sex steroids, menstrual phase

The hormonal regulation of proliferation in the normal breast is controversial and incompletely understood. Estrogen is generally accepted as a promoter of breast epithelial cell proliferation and also thought to be involved in the development and growth of breast cancer. The controversy concerns the action of progesterone-progestogen, for which the literature offers a huge number of conflicting results. Most in vitro studies of breast cancer cell lines and cultured normal cells¹ show progesterone-progestogen to have an inhibitory effect on proliferation, whereas some indicate an independent, additive or synergistic effect in combination with estrogen.²⁻⁴ In vivo experiments so far have been mainly analyses of mitosis in tissue sections from reduction mammoplasties or from "normal" breast tissue near a benign or malignant lesion.⁵ Studies that are based on such tissue specimens have apparent limitations, and

only one proliferation analysis is possible. Most but not all of these studies have shown the highest mitotic activity during the luteal phase.⁵⁻⁸

Fine needle aspiration biopsy is an established technique for the preoperative diagnosis of palpable lumps in the breast. Aspirated cells can also be used for immunocytochemical analyses of estrogen and progesterone receptor content. The development of monoclonal antibodies against cell proliferation specific antigens also has made it possible to assess proliferation in cytologic breast cell samples.

The Ki-67/MIB-1 monoclonal antibody reacts with a human nuclear antigen that is present in proliferating cells but absent in quiescent cells. Cell cycle analysis showed that the antigen is expressed in G₁, S, G₂, and mitosis.⁹ The most significant limitation to the use of Ki-67 has been the need for fresh- or snap-frozen material because the antigen is highly sensitive to fixation. Earlier efforts to assess proliferation in fine needle aspiration biopsies have been hampered by the often low cellularity in cytologic smears.

The MIB-1 antibody, which can be used on paraffin-embedded tissue sections and also on cytospin samples with a higher cellularity than conventional smears, has made it possible to evaluate proliferation in fine needle aspiration biopsies from healthy women.

In this study fine needle aspiration was used together with MIB-1 to assess epithelial cell proliferation in nor-

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mal breast tissue from healthy women during the follicular and luteal phases of the menstrual cycle.

Subjects and methods

Subjects. Forty-nine healthy volunteers were recruited for breast cell aspiration. Two women were excluded because of benign palpable lumps. The remaining 47 women (mean age 32 years, range 18 to 52 years) had regular menstrual cycles of 25 to 35 days and no history or symptoms of any breast disease. All the women had normal breasts on clinical examination. Twenty-seven of the women had previously been pregnant and 23 had children. Twenty-nine of the women were <35 years old. None of the women had taken any sex steroid-containing drugs during the last 6 months preceding the study. Percutaneous fine needle aspiration biopsy was performed during the early follicular phase (days 3 to 5) and midluteal phase (days 20 to 32) according to cycle length, corresponding to days 23 to 25 of a 28-day menstrual cycle. Venous blood samples were drawn the days of biopsy, and testosterone, sex hormone-binding globulin (SHBG), insulin-like growth factor 1 (IGF-1), androstenedione, estradiol, progesterone, and prolactin were analyzed.

Serum concentrations of testosterone were determined by radioimmunoassay (RIA) after extraction with diethyl ether with a commercial kit (Testosterone Double Antibody) obtained from Diagnostic Products Corp., Los Angeles. Serum androstenedione was determined by RIA after extraction with diethyl ether by a method developed at our departments.¹⁰ SHBG and IGF-1 concentrations were determined by RIA with commercial kits obtained from Eurodiagnostics AB, Malmö, and Nichols Institute Diagnostics, San Juan Capistrano, California, respectively. IGF-1 was extracted from serum with acid ethanol before analysis.

The respective detection limits and intraassay and interassay coefficients of variation were as follows: testosterone, 0.13 nmol/L, 8%, and 10%; androstenedione, 0.6 nmol/L, 6%, and 10%; SHBG, 0.05 nmol/L, 4%, and 8%; IGF-1, 0.6 µg/L, 6%, and 10%.

Estradiol, progesterone, and prolactin were analyzed by routine hospital methods.

Forty-two of the women underwent aspiration twice consecutively during the same menstrual cycle, one in the follicular phase only and four in the luteal phase only. Of the 46 women with luteal phase aspirates and blood samples, 28 had hormonally confirmed ovulation (serum progesterone level >16 nmol/L). The study was approved by the local ethics committee, and all women gave their informed consent to participate. Institutional review board approval was obtained June 11, 1990, and Feb. 21, 1994 (KS 94-98).

Fine needle aspiration biopsy. Percutaneous fine needle aspiration biopsy was performed with a needle with

an outer diameter of 0.6 mm as described by Franzén and Zajicek.¹¹ To produce several identical slides the aspirated cells were mixed with 0.5 ml of 4% buffered (pH 7.4) formalin in the same syringe as the procured cells. Volumes of 110 µl were cytocentrifugated at 700 revolutions/min for 3 minutes, and enriched epithelial cells were spotted on pretreated glass slides.

Immunocytochemical receptor analysis. The MIB-1 analyses were performed with reagents supplied by Immunotech, Marseille. The staining procedure uses an avidin-biotin peroxidase system, modified for the cytospin technique. The immunocytochemical receptor analysis stainings were scored and evaluated as previously described.¹² On average, aspirates yielded 400 to 600 cells per slide, and in all cases a minimum of 50 epithelial cells was scored. We considered samples obtained by fine needle aspiration to be assessable only if they contained intact epithelial cells and not free-lying nuclei.

Statistical analysis. Differences were assessed by the Wilcoxon rank sum tests for paired and unpaired observations. For correlation analyses Spearman's rank correlation coefficient was calculated.

Results

Out of a total number of 89 aspirates 72 were evaluable for MIB-1 content, 34 from the follicular phase and 38 from the luteal phase. The remaining aspirates were non-evaluable because of low cellularity. The distribution of MIB-1 values during the menstrual cycle is illustrated in Fig. 1. There was skew distribution of the proportion of MIB-1-positive cells in the follicular phase. In a majority of cases (75%) proliferation was ≤2%. The distribution of luteal phase values was clearly different with 16 of 38 cases exceeding 2%. The percentage of MIB-1-positive cells of all evaluable samples tended to be higher in the luteal phase (mean 2.04%, median 2%, range 0% to 6%) than in the follicular phase (mean 1.66%, median 1%, range 0% to 7%) phase. This difference did not reach statistical significance. In the 29 women aged <35 years there were significantly higher MIB-1 values in the luteal phase (mean 2.29%, median 2%, range 0% to 6%, $n = 24$) than in the follicular phase (mean 1.13%, median 0.5%, range 0% to 6%, $n = 23$) ($p = 0.003$), as shown in Fig. 2.

Of the 42 women who underwent aspiration twice in the same menstrual cycle, 25 had evaluable samples from both phases. In these women (mean age 32 years, range 21 to 52 years) the mean percentage of MIB-1-positive cells increased (from mean 1.3%, median 1%, range 0% to 7% in the follicular phase to mean 2.1%, median 2%, range 0% to 6% in the luteal phase) ($p = 0.05$). Eighteen women (mean age 32 years, range 25 to 52 years) had two adequate aspirates and also hormonally confirmed ovulation. Here the follicular phase value was mean 1.3%, median 0.75%, range 0% to 6% and the luteal phase

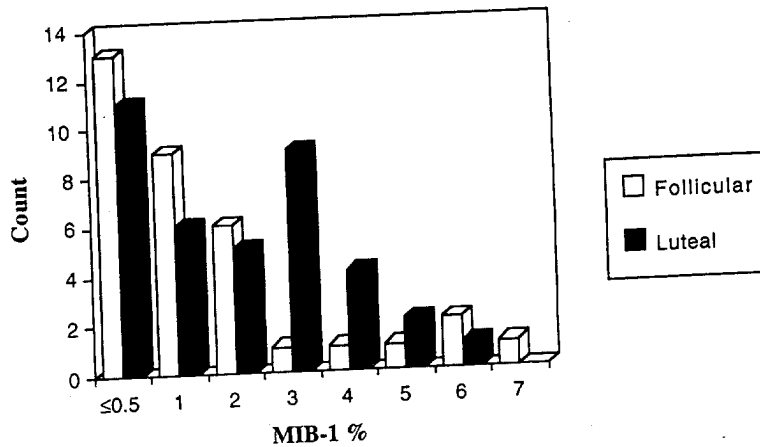


Fig. 1. Distribution of percentage of MIB-1-positive cells in early follicular ($n = 34$) and luteal ($n = 38$) phase of menstrual cycle.

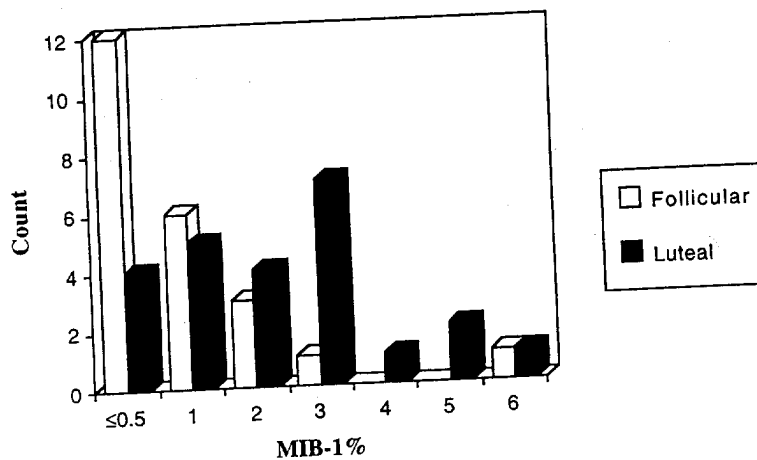


Fig. 2. Distribution of percentage of MIB-1-positive cells in early follicular ($n = 23$) and luteal ($n = 24$) phase in women <35 years old.

value was mean 2.4%, median 2.25%, range 0% to 6%) ($p < 0.04$, Fig. 3).

A correlation analysis between sex hormone levels and proliferation was done for all the serum hormones analyzed. There was a significant positive correlation in the total material between serum progesterone levels the day of aspiration and MIB-1 values ($r_s = 0.23$, $p < 0.05$). The significance of this correlation was enhanced in women aged <35 years ($r_s = 0.47$, $p = 0.002$) and in the 25 women with two evaluable samples ($r_s = 0.35$, $p < 0.02$). In the smaller group of 18 women with confirmed ovulation the correlation remained significant ($r_s = 0.34$, $p < 0.05$).

There were no significant correlations between estradiol, prolactin, testosterone, IGF-1, androstenedione, SHBG, testosterone/SHBG ratio, or IGF-1/SHBG ratio, on the one hand, and the percentage of MIB-1-positive cells, on the other. This was true both for the total material from the two phases and from each phase separately.

There was a trend for a positive correlation between age and follicular phase MIB-1 values for the 34 women with an adequate aspirate in this phase ($r_s = 0.28$, $p = 0.09$). There was no correlation between age and luteal phase MIB-1 values or age and the individual mean MIB-1 values calculated from the two samples. In the 25 women with two adequate aspirates there was a highly significant positive correlation between age and follicular phase MIB-1 values ($r_s = 0.51$, $p = 0.01$).

Comment

To our knowledge this is the first report on proliferation assessed by fine needle aspiration biopsies from normal breasts of healthy women. This was accomplished by the introduction of the MIB-1 antibody in combination with the cytospin technique, which uses the aspirated cells more effectively than the conventional smear technique.

An increased proliferation in the luteal phase was clearly demonstrated in women aged 21 to 52 years with

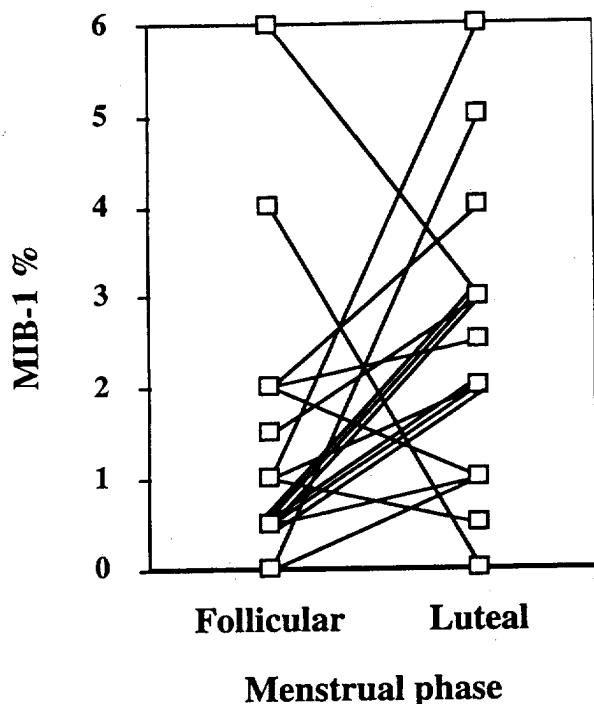


Fig. 3. Change in percentage of MIB-1-positive cells in follicular and luteal phase in women ($n = 18$) with two consecutive fine needle aspiration biopsies in same menstrual cycle and with hormonally confirmed ovulation.

two evaluable samples in the same menstrual cycle and also in those with a hormonally confirmed ovulation. In the total material, when all single samples were included, there was a higher proliferation in the luteal phase, which was significant in women <35 years old. This is in line with most previous *in vivo* studies on tissue material from reduction mammoplasties and near fibroadenomas.^{5,7} The increase in mitotic activity during the luteal phase suggests a proliferative action of progesterone. Progesterone has previously been reported to either increase, decrease, or have no effect on mitotic activity and proliferation in breast epithelial cells. To what extent addition of progestogen affects proliferation and cancer risk during hormone replacement therapy is highly controversial.

Our data show a significant positive correlation between proliferation and serum progesterone level recorded the day of aspiration. The correlation was apparent for the total material, also including single samples, and further enhanced in women <35 years old and in those with two successful aspirates during the same menstrual cycle.

Recent data from an experimental *in vivo* model for hormone replacement therapy render further support for a proliferative action of progestogens. In surgically postmenopausal *Cynomolgus* macaques, combined estrogen-progestogen treatment caused more proliferation

and hyperplasia than treatment with estrogen alone.¹³ These primates, unlike rodents, have a cytokeratin subtype reactivity similar to that of humans; a 28-day menstrual cycle, and hormonally defined follicular and luteal phases.

An important question for the interpretation of fine needle aspiration data is the possibility of regional differences in breast epithelial proliferation. For obvious reasons studies on this issue are difficult to perform on normal breast tissue from healthy women. However, in the mammary gland of surgically postmenopausal macaques we found no differences in proliferative activity between alveolar and ductal structures.¹³ Furthermore, we investigated proliferation in 10 different locations of the macaque breast and found no significant differences between quadrants or by distance from the nipple (unpublished data).

Whereas estrogens are clearly mitogenic for breast epithelial cells, the effects of progestogens are more complex. In the endometrium progestogens inhibit estrogen-induced cell cycle progression early in the G_1 phase, whereas in the breast progestogens may both stimulate and inhibit proliferation.^{14, 15}

A direct proliferative effect of progestogens on breast cancer cell lines has been observed in many recent *in vitro* studies when phenol red-free media are used.^{2,4, 16} However, when the same contraceptive progestogens that induced proliferation in breast cancer cell lines were used in an estrogen-dependent DMBA rat breast cancer model, these progestogens hampered tumor progression.³ Both this group³ and Jeng et al.¹⁶ found that antiestrogen but not antiprogestogen counteracted the proliferative effect of progestogens on breast cancer cell lines. The latter authors also showed that the proliferative effect of progestogen was mediated through the estrogen receptor and not the progesterone receptor by using cells transfected with a *CAT* reporter gene containing an estrogen response element. Reports of different isoforms of the progesterone receptor and observations that several growth factors (e.g., IGF-1, bovine fibroblast growth factor, and epidermal growth factor) may also, in the absence of sex steroids, initiate cell cycle progression further complicates the picture.^{14, 15, 17} Pretreatment with progesterone was found to reduce breast epithelial cell proliferation in women undergoing breast surgery.¹⁸ However, the tissue concentrations of estradiol and progesterone in that study markedly exceeded the physiologic range.¹⁹ Also in human breast xenografts in athymic nude mice estradiol but not progesterone increased proliferation.²⁰ Tentatively this discrepant finding could be related to differences in paracrine and hormonal influence. Adjacent fat tissue is important for the *in vivo* regulation of breast proliferation.^{21, 22} Tissue estrogen receptor content was remarkably low and not affected by progesterone treatment.

We previously demonstrated that progesterone receptors in the breast, in contrast to the endometrium, are maintained and expressed at high levels during the luteal phase.²³ According to experiments in the rat, progestogens, acting via estrogen-dependent progesterone receptor, could promote breast epithelial cell proliferation.²⁴ Furthermore, thymidine kinase, an enzyme of the nucleotide synthesis considered to be an important marker of cell growth, is stimulated at physiologic progesterone concentrations.² Insulin receptor content and insulin stimulation of cell growth also are enhanced by progestogens in human breast cancer cell lines.⁴ These data indicate a direct stimulating action of progestogens on the breast. In the macaque study long-term estrogen-progesterone treatment induced marked proliferation despite down-regulation of both estrogen and progesterone receptors.¹³

Several other hormones including estradiol, androgens, prolactin, SHBG, and IGF-1 have been reported to influence proliferation and cancer risk.^{2, 4, 25} In the current study none of the serum concentrations of these factors displayed any significant correlation with proliferation. It is possible that more frequent blood sampling throughout the cycle had revealed other correlations.

Interestingly, there was a significant positive correlation between age and follicular phase proliferation rate. This is in contrast to the data from Anderson et al.,⁵ who found a lower proliferation rate with increased age, but they did not analyze cycle phases separately. In four individual women >35 years old we found a remarkably high proliferation rate (4% to 7%) during the follicular phase. In three of these women with a successful second aspiration, proliferation was suppressed during the luteal phase (0% to 3%). Thus these women apparently differed from the rest of the study group. However, there was no sign of hormonal or breast abnormality in these women. Further studies of the possible existence of a subgroup with diverging regulation of proliferation are clearly indicated.

In conclusion, the fine needle aspiration biopsy technique is a valuable tool for in vivo studies of cell proliferation in the normal breast. Basic understanding of breast proliferation and response to sex steroids is crucial for the interpretation of conflicting epidemiologic data regarding cancer risk and hormone treatment. We found a higher proliferation during the luteal phase and a positive correlation with serum progesterone levels. Data clearly suggest a proliferative action of progesterone. Studies of women on a regimen of oral contraceptives or hormone replacement therapy are in progress.

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