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Proliferation of Normal Breast Epithelial Cells as Shown by *In Vivo* Labeling with Bromodeoxyuridine

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The proliferative activity of normal acinar and ductal breast epithelial cells was studied by* in vivo *labeling with 5-bromodeoxyuridine (BrdUrd) in 26 cases with concurrent breast carcinoma. The BrdUrd-labeled cells were recognized in histologic sections of paraffin-embedded tissue, using an anti-BrdUrd antibody and an immunoperoxidase reaction. The percentage of BrdUrd-labeled cells showed great variability for both acinar (0% to 2.66%; mean, 0.70%; standard deviation [SD], 0.80%) and ductal cells (0% to 1.99%; mean, 0.51%; SD, 0.57%). The fraction of proliferating epithelial cells declined with the age of the patients and was significantly higher in premenopausal women (1.16% ± 0.85% for acinar and 0.94% ± 0.60% for ductal cells) as compared with the postmenopausal women (0.27% ± 0.46% for acinar and 0.17% ± 0.22% for ductal cells), $P < 0.01$ for acinar and $P < 0.001$ for ductal cells, respectively. In some patients, great variability in distribution of proliferating acinar and ductal cells among different lobules and ducts was observed. No difference was found in the number of proliferating acinar and ductal cells situated near or far from their corresponding tumors. No correlation was seen between cell proliferation of normal acinar or ductal cells and cell proliferation of the respective tumors. (Am J Pathol 1991, 138:1371–1377)

Several studies have characterized cell proliferation of breast carcinomas.[1–5] Little information is available, however, on the proliferation of normal epithelial and nonepithelial cell populations in human breast tissue. Until recently, all data published on the cell proliferation of human mammalian cells were obtained either by evaluating the number of mitoses present, or by counting labeled cells after *in vitro* incubation with ^{3}H thymidine.[6–9] The *in vitro* studies with ^{3}H thymidine indicated that cell proliferation of the breast epithelial cells depends on many factors: age of the patients, hormonal status, menstrual cycle, antiestrogen treatment, and pituitary activity.[10–14] In most of these studies it was shown that the highest values for ^{3}H-thymidine labeling index occurred during the last week of the menstrual cycle.[12,15]

In this study we present data on the proliferative activity of normal breast epithelial cells after *in vivo* labeling of cellular DNA with 5-bromodeoxyuridine (BrdUrd) to identify DNA-synthesizing cells. This technique uses a monoclonal antibody against BrdUrd and the immunoperoxidase reaction. 5-Bromodeoxyuridine labeling also is used to evaluate proliferation in acinar and ductal cells. Labeling of the normal cells is then compared with labeling of the corresponding malignant tumors.

Material and Methods

Patient Selection

Normal breast tissues were collected from 26 women aged 25 to 79 years who were undergoing partial mastectomy for breast carcinoma. 5-Bromodeoxyuridine, 200 mg/m^2, was injected intravenously into patients 30 minutes before surgery.[16] Written informed consent was obtained from all patients. Fourteen of the patients were

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postmenopausal and 12 premenopausal. All postmenopausal women had ceased having periods for at least 1 year before surgery. Six premenopausal patients gave information on their menstrual cycle and the use of contraceptive hormones. The day of the menstrual cycle was calculated by counting from onset of the last menstrual bleeding to the day of surgery.

Tissue Sampling and Staining

Normal tissue samples were taken from surgical specimens approximately 1 to 2 cm from the tumor nodule. Tissue was fixed in 70% ethanol or in 10% pH 7, sodium phosphate-buffered formalin. After embedding in paraffin, sections were cut and stained by hematoxylin and eosin for determination of cellular components in the tissue block. Parallel sections were used for visualization of BrdUrd-positive cells. For BrdUrd staining, paraffin sections were placed in a 60°C oven for 30 minutes; deparaffinized in xylene twice for 3 minutes; hydrated in graded ethanol, 100% and 95% twice each for 3 minutes; and washed in water. Endogenous peroxidase was quenched with 1% H_2O_2 for 15 minutes. The slides were washed further with double distilled water, incubated in 2 N HCl for 60 minutes at room temperature to denature double-stranded DNA, and washed with distilled water and phosphate-buffered saline (PBS). Formalin-fixed samples were treated for 1 minute with 0.1% protease (Serva Co., Uppsala, Sweden), and again washed with water and PBS. The sections were incubated with IU4 primary antibody (Lawrence Livermore National Laboratory, Livermore, CA), diluted 1:10,000 in PBS and 2% horse serum for 1 hour at 37°C, followed by biotinylated horse anti-mouse antibody (1:500 in PBS) for 45 minutes at room temperature, washed with PBS, incubated in ABC reagent (Avidin-Biotin Complex; Vector Laboratories, Burlingame, CA) for 10 minutes, and stained with diaminobenzidine for 10 minutes.[16] Light counterstaining with hematoxylin was used to visualize nuclei.

Scoring of Labeled Cells

The number of cells per cross-sectioned acinus was determined in 12 cases. This number varied between 75 and 1690 cells per acinus. In this report the term 'acinus' is used for the terminal units seen within the breast during the resting stage. The term 'ductal' cells should be understood to mean the epithelial cells (luminal and myoepithelial) that line the intralobular and extralobular ducts. Cells in the intralobular terminal ducts as well as in the larger interlobular ducts were counted separately from the acinar cells. The myoepithelial cells were scored together with the epithelial cells. The BrdUrd-labeled cells were recognized easily by their brown staining. A minimum of 1500 ductal and 1500 acinar cells were scored per case. The labeling index (LI) was defined as the number of BrdUrd-positive cells divided by the total number of cells counted expressed as a percentage. To estimate the interlobular variability in the number of proliferating acinar cells, BrdUrd-labeled cells per lobule also were scored in cases with a high number of proliferating cells. Estimation of the percentage of labeled cells was done without any knowledge of clinical and menstrual status. Statistical comparisons of labeling indices were performed using *t*-test and linear regression analysis.

Results

The brown-colored reaction product of BrdUrd-stained cells was localized over the nucleus. In most postmenopausal women the lobular acini appeared regular and were lined by a single layer of epithelial cells (Figure 1a). In premenopausal women, variability in the size and cellular composition of the acini and ducts was observed (Figures 1b, c). In addition, epithelial and stromal components showed changes corresponding to the phases of the menstrual cycle. No mitotic figures were observed among the group of postmenopausal women, but occasional mitoses were found among the group of premenopausal women.

For the 26 cases examined, the BrdUrd labeling indices ranged between 0% (even after scoring more than 5000 cells) and 2.66% for acinar cells, and between 0% and 1.99% for ductal cells (Table 1). No ductal epithelial cells were found in case 209 and no acinar epithelial cells were found in case 215. As the table shows, there was great variability in the percent of BrdUrd-labeled acinar and ductal cells, both among premenopausal and among postmenopausal women. In two postmenopausal women (cases 142 and 206), the BrdUrd LI of acinar cells were even higher than in most premenopausal women. No significant difference was found between the LIs of acinar and ductal cells among the whole group of patients, however ($P = 0.13$, paired *t*-test), and a relatively good correlation was observed ($r^2 = 0.64$) between the LI of acinar and ductal cells (Figure 2).

Examples of lobular units having relatively low or high numbers of BrdUrd-labeled acinar and duct cells are shown in Figures 1a and 1b, respectively. In some cases, great variability in distribution of labeled cells was seen. Thus some lobuli were observed with a high number of labeled cells, whereas other lobuli from the same case lacked labeled cells. Within some lobuli, the distribution of

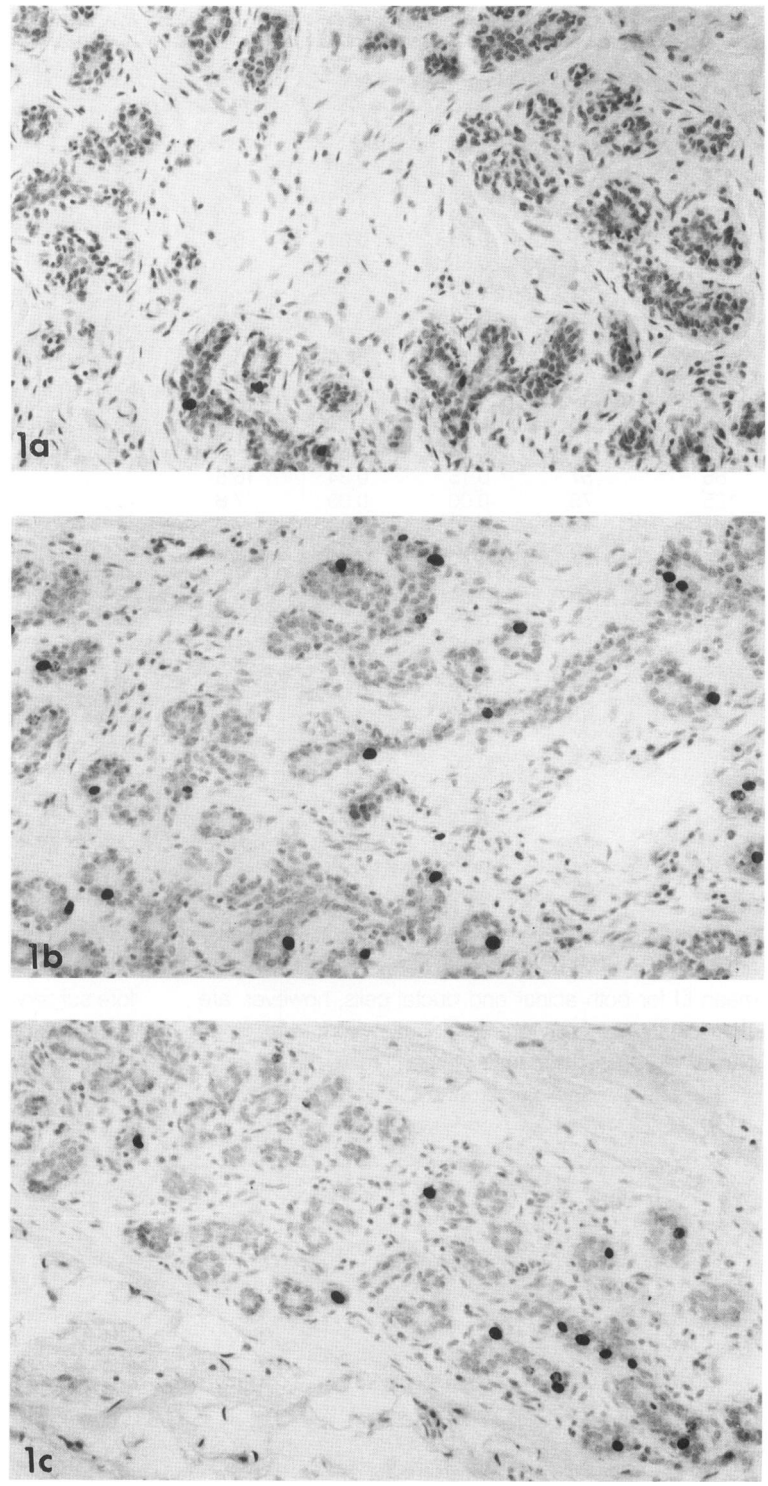

Figure 1. *Photomicrographs (×100) of normal breast tissues, labeled* in vivo *with BrdUrd and stained with H&E. BrdUrd-labeled nuclei are identified by the immunoperoxidase reaction using IU4 antibody.* **a**: *Postmenopausal woman (case 152) showing few labeled nuclei (acinar LI = 0.1%; lobular LI = 0.2%).* **b**: *Premenopausal woman (case 145) showing more labeled nuclei (acinar LI = 1.9%; lobular LI = 1.2%).* **c**: *Premenopausal woman (case 161) showing a heterogeneous distribution of labeled nuclei within a single lobular structure (acinar LI = 1.9%; lobular LI = 1.1%).*

labeled cells also was heterogeneous (Figure 1c). To obtain more information about the variability in distribution of BrdUrd-labeled cells among lobuli, all the cells in an entire lobule were counted and the LI for each lobule was evaluated in each of eight cases (Figure 3). Note the large range (~5×) in the LI for the lobules from each of these eight cases.

The distribution of BrdUrd labeling indices as a function of the age of the patients is given in Figure 4. There is no significant correlation between the age of the patients

Table 1. *BrdUrd Labeling Indices of the Breast Epithelial Cells*

Case no.	Age	LI-% Acini	LI-% Ducts	LI-% Tumors
Premenopausal				
126	47	2.66	1.30	14.4
137	35	0.62	0.26	17.2
145	40	1.89	1.20	4.0
155	31	0.54	0.94	0.9
156	47	0.59	0.60	3.7
161	46	1.89	1.10	0.3
165	42	1.14	1.60	5.6
169	34	0.05	0.05	1.0
178	44	0.12	0.26	0.9
186	25	0.75	1.07	14.4
187	47	2.21	1.99	20.9
209	46	1.48		21.6
Postmenopausal				
86	57	0.15	0.34	16.5
125	75	0.00	0.00	7.8
127	49	0.10	0.00	7.9
135	63	0.00	0.00	4.9
141	79	0.05	0.09	1.6
142	69	1.04	0.10	5.6
150	61	0.09	0.23	1.3
152	69	0.12	0.22	4.1
153	62	0.17	0.13	9.6
171	71	0.13	0.20	0.7
177	48	0.12	0.26	2.8
206	46	1.50	0.00	11.1
208	65	0.00	0.00	9.1
215	80		0.80	9.7
Mean ±SD	54 ± 15	0.70 ± 0.80	0.51 ± 0.57	7.59 ± 6.48

and the percentage of proliferating cells (r^2 = 0.056 for acinar and r^2 = 0.023 for ductal cells, respectively). The mean LI for both acinar and ductal cells, however, are significantly higher in premenopausal women as compared with postmenopausal women (Table 2).

The effect of the menstrual cycle on LI was investigated using the six patients who gave a reliable menstrual history. In three women with surgery during the last week of their menstrual cycle (cases 145, 161, 209), the percentage of BrdUrd-labeled cells (LI = 1.89%, 1.48%, 1.89%, respectively) was higher than in patients with surgery between the 6th and 19th day of the menstrual cycle (cases 137, 156, 186) (LI = 0.62%, 0.59%, 0.75%, respectively).

Five patients (cases 156, 161, 167, 186, 209) had been taking contraceptives between 5 and 20 years before surgery. The percent of BrdUrd-labeled cells was in the range of those without contraceptives (Table 1).

In seven cases, areas were selected that were situated far from (at least 2 cm) or close to (within 1 cm) the

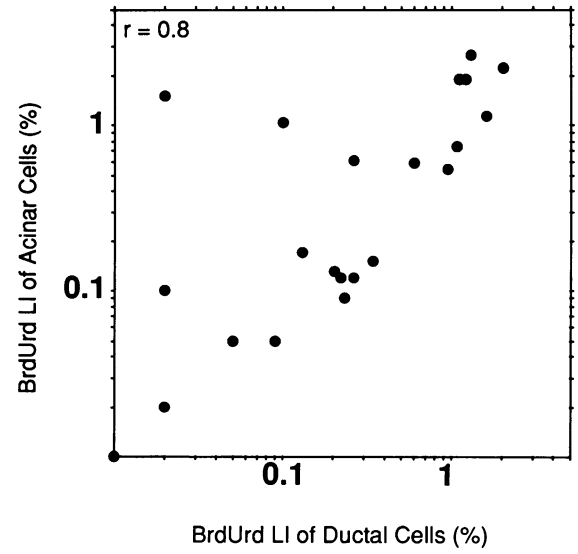

Figure 2. *Comparison, for 24 women, of BrdUrd labeling indices (%, logarithmic scales) of acinar and ductal cells in the same tissue specimens.*

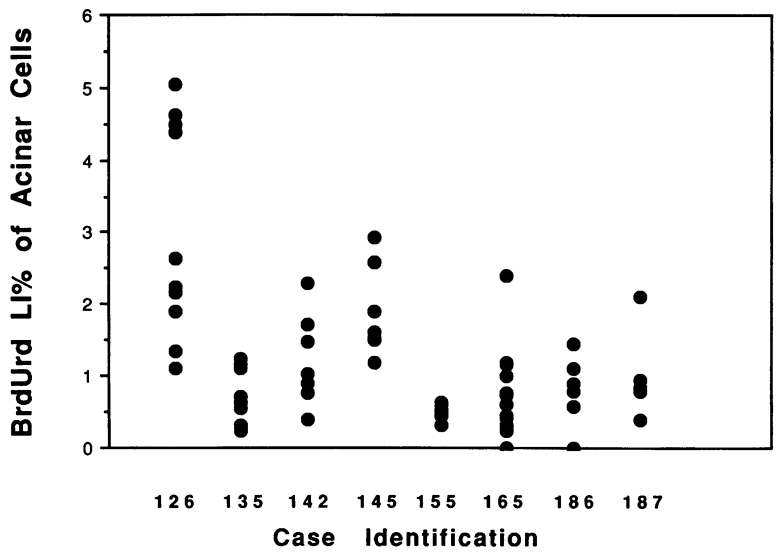

Figure 3. *Distribution, for eight cases, of BrdUrd labeling indices (%, linear scale) of acinar cells in different lobules.*

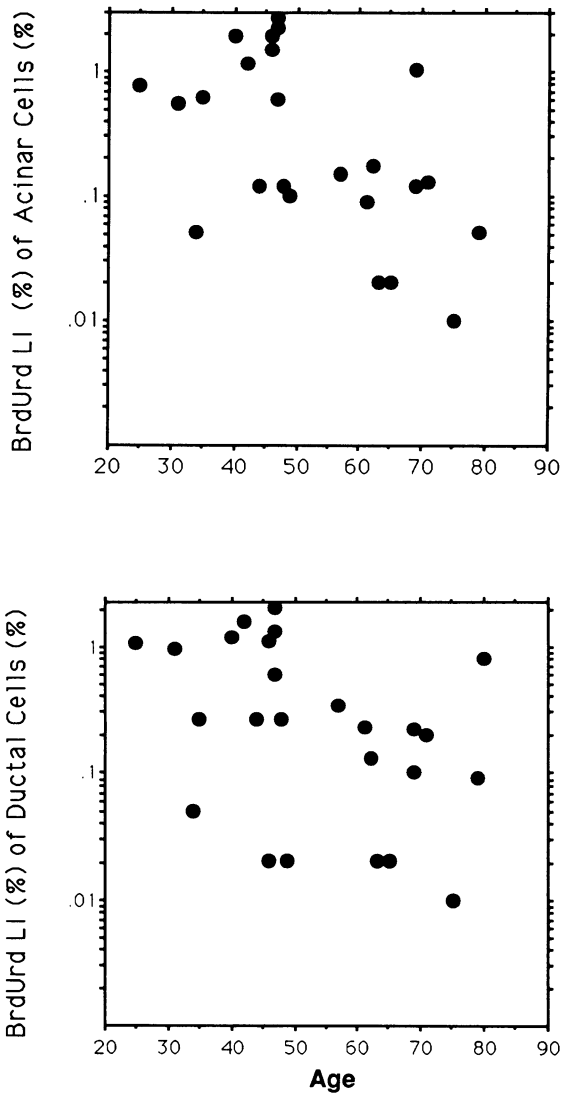

Figure 4. *Relationship between BrdUrd labeling index (%, logarithmic scale) for acinar cells (upper panel) or ductal cells (lower panel) and patient age.*

corresponding tumor, and the BrdUrd LI was determined in the acini and ducts. No significant difference was found in the LI of cells situated far or near to the tumors ($P > 0.1$ for both acinar and ductal cells; Table 3).

Table 2. *Proliferation of Normal Breast Epithelial Cells:* In Vivo *Labeling with BrdUrd (mean ± SD)*

Hormonal status	No.	Ductal		No.	Acinar	
Premenopausal	11	0.94 ± 0.60	$P < 0.001$	12	1.16 ± 0.85	$P < 0.01$
Postmenopausal	14	0.17 ± 0.22		13	0.27 ± 0.46	
Total and mean	25	0.51 ± 0.57		25	0.70 ± 0.80	

Table 3. *Proliferation of Normal Breast Epithelial Cells: Near and Far from Tumor (mean ± SD)*

Situated	No.	Ductal	Acinar
Near	7	0.74 ± 0.88	0.74 ± 0.77
Far	7	0.50 ± 0.41	0.61 ± 0.60
Mean:		0.62 ± 0.67	0.67 ± 0.67

Along with the percentage of proliferating normal epithelial cells we had evaluated the percentage of BrdUrd-labeled cells in the corresponding breast carcinomas. No significant correlation was found between the values of BrdUrd-labeled normal epithelial cells and tumor cells ($r^2 = 0.149$ for acinar and $r^2 = 0.074$ for ductal cells, respectively).

Discussion

This study is part of a research project devoted to the investigation of cellular and molecular markers of malignancy in breast tumors.[16–18] Here attention is paid predominantly to the proliferation of normal epithelial acinar and ductal cells. Proliferating cells were labeled by an *in vivo* procedure that allows all cells replicating DNA to incorporate BrdUrd.[16] BrdUrd substitutes for thymidine in DNA synthesizing cells during the S-phase of the cell cycle.

Most data published until now on the proliferation of normal and malignant breast tissues have been obtained from studies using a variety of *in vitro* labeling procedures. *In vitro* procedures for labeling proliferating cells are quite variable, however (with or without hyperbaric oxygen, different cell culture media, different times of incubation, variability in the thickness of tissue blocks, mechanical or enzymatic disintegration of tissue samples, etc.) so that data obtained by different authors are difficult to compare. The main area of doubt in using tissue blocks is whether or not ^{3}H thymidine or BrdUrd under *in vitro* conditions penetrate in an appropriate concentration to all proliferating cells. This problem is particularly important for normal breast tissues where the epithelial cells are usually surrounded by noncellular connective tissue components. Studies by Meyer and Connor,[19] however, using *in vitro* labeling of breast tissue samples with ^{3}H thymidine, showed values for labeled lobular and ductal cells both in premenopausal and postmenopausal women that are quite similar to our results using an *in vivo* protocol for labeling of proliferating cells with BrdUrd.

Our data show that proliferative activity of normal acinar and ductal cells is quite variable for both premenopausal and postmenopausal women. The percentage of BrdUrd-labeled cells is significantly higher in premenopausal women than in postmenopausal women, however

($P < 0.01$ for acinar cells and $P < 0.001$ for ductal cells; Table 2). These results are in the range of other studies using ^{3}H thymidine for labeling of proliferating cells.[6,8,9] With our technique, proliferating cells were found among the group of postmenopausal women. Evidence of postmenopausal proliferation was obtained also by Russo et al,[14] who used thymidine labeling *in vitro*.

Data obtained in our study also indicate that there is no difference in the proliferative activity of acinar and ductal epithelial cells. Joshi et al,[8] by means of ^{3}H thymidine incorporation, also did not find a significant difference between the LI of lobular and ductal cells. They indicated, however, that the number of proliferating epithelial cells is higher than those of myoepithelial cells.

In some of the cases, attention was paid to the heterogeneity in intralobular and interlobular distribution of BrdUrd-labeled cells. Our data presented in Figure 3 show that in normal breast tissue there are lobules and even single acini that have a higher labeling index than other areas. By the *in vivo* approach for labeling of proliferating cells with BrdUrd in this study, it was possible for large tissue samples to be examined and for heterogeneity in distribution of proliferating cells to be easily observed. With *in vitro* labeling of small tissue blocks with ^{3}H thymidine or BrdUrd, it is more difficult to estimate the heterogeneity in proliferative activity of normal epithelial cells. Even in small tissue samples incubated *in vitro* with ^{3}H thymidine, however, variability in distribution of labeled cells has been observed.[19] It remains unclear whether or not patients with greater proliferation in certain breast regions might be considered as a group at increased risk to develop malignancy.

Most previous studies have shown that the number of proliferating breast epithelial cells increases during the last week (days 21 through 28) of the menstrual cycle.[6,9,14] Our study supports these observations, although the number of cases is small. Five patients had taken oral contraceptives for long periods (between 5 and 20 years), but the percentage of BrdUrd-labeled acinar and ductal cells show no significant differences from patients without hormonal treatment. The results of Meyer[6] and Potten et al[15] also indicated no significant difference in proliferating cells between women taking oral contraceptives and those not. In a recent publication, however, Anderson at al[13] reported an increased number of proliferating breast epithelial cells in nulliparous women who had been using oral contraceptives for a long time. It is evident that additional studies are needed to clarify the effect of oral contraceptives on the proliferative activity of the human breast.

The lack of differences in the percentage of BrdUrd-labeled cells among the tissue samples situated near or far from tumors as well as the lack of correlation between the number of proliferating cells in normal tissue and malignant tumors suggests that tumor cells do not directly affect normal epithelial breast cells, at least as far as their proliferative activity is concerned. Thus we find no evidence to support the concept of tumor-induced proliferative activity or field effect.

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