Nonrandom Frequency Distribution of Mitoses in Rat Lobuloalveolar Mammary Gland Epithelium

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A quantitative microscopic technique was employed to examine the distribution of mitotic activity in the rat mammary gland. The frequency distributions of mitoses per unit volume of lobuloalveolar mammary gland epithelium in virgin Lewis/Mai rats at each phase of the estrous cycle were determined and compared to the expected Poisson frequency distributions, assuming random mitotic activity. Both pooled data and data from individual rats were compared to expected Poisson distributions. At each phase of the estrous cycle, the pooled observed distributions deviated significantly from Poisson distributions. Sixty-seven percent (72/108) of the observed frequency distributions obtained from individual rats also deviated significantly from expected Poisson distributions. These data indicate a nonrandom distribution of mitoses in rat lobuloalveolar mammary gland epithelium. This observation suggests that local cell products and/or a variation in the extent of replicative synchrony of lobuloalveolar cell populations may determine in part the pattern of mitotic activity in this tissue. A nonrandom distribution of mitoses in mammary epithelium may have significance in relation to the genesis of hyperplastic and neoplastic lesions of the mammary gland. (Am J Pathol 88:267–276, 1977)

LOCALIZED FOCI OF MITOSES have been noted in a variety of epithelial tissues,[1-10] an observation which suggests that mitoses may have a nonrandom tissue distribution. Although statistical analyses of such apparently nonrandom mitotic distributions have been uncommon, some studies [6-10] have presented statistical evidence to support this conclusion.

In a previous study, we showed that the frequency distribution of mitotic activity in lobuloalveolar tissue of the female Lewis/Mai rat mammary gland was heterogenous at each phase of the estrous cycle.[11] The heterogeneity of mitotic activity in the mammary gland raised the possibility that mitosis was occurring nonrandomly in this tissue. Because a nonrandom distribution of mitoses would have important implications for the control of mitotic activity in the mammary gland, we have investigated this question further.

In this report, our previous data and additional new data are analyzed

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statistically to determine the nature of the distribution of mitoses in the rat mammary gland. At each stage of the estrous cycle the pooled frequency distribution of mitoses in lobuloalveolar tissue differed significantly from that predicted on the basis of a Poisson distribution. A similar finding was, in most cases, also obtained when mitotic frequency distributions from individual rats were compared to expected Poisson distributions. These data show that the distribution of mitoses in the rat mammary gland is nonrandom. This observation suggests that local cell products (mitotic stimulators) and/or variation in the degree of replicative synchrony of lobuloalveolar cell populations may determine in part the pattern of mitotic activity in this tissue.

Materials and Methods

Animals

Female Lewis/Mai rats were obtained from Microbiological Associates, Inc., Bethesda, Md. Rats were housed 5 animals/cage, in proximity to cages of male rats and under a light/dark cycle of 12 hours/12 hours.

Vaginal Cytology

Vaginal smears were obtained from all rats in the mornings for 1 week prior to beginning the experiments to ensure regularity of estrous cycles. Vaginal smears were stained by the method of Papanicolaou and conventional cytologic criteria used to assign phase of the estrous cycle. Rats used in these experiments had regular 4-day estrous cycles. In experiments, vaginal smears were obtained at the time of injection of colchicine and at the time rats were killed for tissue collection.

Mitotic Blockade

In the experiments, virgin postpubertal rats 60 to 90 days of age were given an intraperitoneal injection of colchicine (Calbiochem, Los Angeles, Calif.), 1 mg/kg body weight, 5 hours prior to killing them with CO_2 in order to arrest mitosis at metaphase. Colchicine was given between 9:00 AM and 10:00 AM, and the rats were killed between 2 PM and 3 PM. Multiple rats were killed in proestrus, estrus, metestrus, and diestrus I and II.

Tissue Preparation

The right inguinal mammary glands were removed from the rats and prepared for sectioning as before.[11] The left inguinal mammary glands were prepared as stained whole amounts for another study. One 25-μ section was cut from each mammary gland parallel to the surface of the mammary gland tree. These sections were stained with Harris's alum hematoxylin, differentiated in acid-alcohol, dehydrated, and mounted under glass in Permount (Fisher Chemical Company, Fairlawn, N.J.).

Microscopic Analysis

Mitotic analysis was carried out using the technique previously described.[11] Twenty-seven to 40 (mean, 38) lobuloalveolar tissue samples were obtained for analysis from each of the mammary gland sections using the following tactic. A transparent numbered grid

(Microlocator slide, Scientific Products, Evanston, Ill.) was placed over the tissue section. Under a magnification of × 2, 40 numbered grid-squares (10 from each quadrant overlying the tissue section) were selected at random and recorded. Under the magnification used, no cellular details of the mammary tissue were visible, which allowed a sample to be selected with no bias as to the degree of mitotic activity present in the tissue. The grid-slide was then placed onto the mechanical stage of a microscope equipped with an eyepiece-grid micrometer, and one of the previously selected grid squares was located. The grid-slide was removed from the microscope stage without moving the position of the stage and replaced with the tissue section. At a magnification of × 400, an area of lobuloalveolar tissue lying within or nearest to the selected area was centered inside of the eyepiece-grid micrometer image. If no lobuloalveolar tissue was encountered, the field was discarded and another of the preselected grid squares located. The microscope was focused up and down, and the number of mitoses and interphase nuclei in the underlying tissue determined and recorded. The area of lobuloalveolar tissue examined was estimated from the proportion of the eyepiece-grid micrometer overlying the tissue. Measurements of tissue thickness were made at the same time. This process was repeated until all of the preselected tissues were analyzed.

Treatment of Data

Data from individual rats within each phase of the estrous cycle were combined to form composite frequency distributions of the lobuloalveolar cell mitotic activity characteristic of each phase of the cycle. From these distributions the mean mitotic activity of lobuloalveolar tissue was calculated and expressed as the number of mitoses/cu mm of tissue. This was done as before [11] by calculating the mean ± SE using each individual mitotic data point for each rat in a given phase of the estrous cycle. For the statistical analysis, data were expressed as the number of mitoses present in a volume of lobuloalveolar tissue underlying ten eyepiece micrometer grid squares. At the magnification employed (× 400), the area of tissue enclosed by ten micrometer grids was 0.0062 sq mm. Mean tissue section thickness was 0.022 ± 0.00 mm (based on 525 point samples from 21 slides) in the first series and 0.028 ± 0.00 mm (based on 192 point samples from five slides) in the second series. Therefore, the mean volume of lobuloalveolar tissue in which mitoses were enumerated was 1.36×10^{-4} cu mm in the first series and 1.74×10^{-4} cu mm in the second series. The total number of nuclei (metaphase + interphase) within these tissue volumes ranged from 101 to 129 in the first series and from 109 to 118 in the second series.

To determine if the observed frequency of mitoses per unit volume of lobuloalveolar tissue in the two series were randomly distributed, we calculated from these data the fitted Poisson frequency distributions [12] which would be expected on the basis of random mitotic activity. Pooled data from individual rats in the two series were compared to expected Poisson distributions. The observed and expected frequency distributions were then compared statistically for goodness of fit using the Chi-square analysis.[13] Distributions were considered different if the observed and expected frequency distributions were different at the $P < 0.05$ level. To substantiate any deviation from randomness observed in the case of pooled data, Poisson distributions were also generated using the data from each rat individually and subjected to Chi-square analysis. This tactic controlled for differences between individual rats which could influence conclusions drawn from pooled data.[10]

Fitted Poisson distributions and Chi-square analyses were carried out with a Hewlett-Packard HP-67 programmable pocket calculator.

Results

The observed frequency distributions of mitoses per unit volume of lobuloalveolar tissue and their corresponding fitted Poisson frequency

distributions for the first series of rats (N = 28; 5 or 6 rats at each cycle phase) are shown in Table 1. The observed distributions deviated from the expected distributions at each phase of the estrous cycle. The deviations were the result of an excess frequency of lobuloalveolar tissue with relatively high densities of mitoses and an excess of mitotically inactive areas in comparison to the Poisson distributions.

In Table 2, Chi-square values indicating degree of fit to Poisson distributions for the pooled data and the Chi-square values obtained for each individual rat contributing to the pooled data are presented. Chi-square values for the pooled data indicate that at each phase of the estrous cycle the observed distribution of mitoses differ significantly from the distribution expected on the basis of random mitotic activity.

With the exception of the proestrus rats, where only 1 rat of 6 showed significant deviation from randomness when individual data were considered, Chi-square values obtained from individual rats of other cycle

Table 1—Observed Frequency (O) and Expected Frequency (E) of Mitoses in Samples of Rat Mammary Gland Epithelium (First Series)—Pooled Data

No. of mitoses/ unit volume = r	Cycle phase									
	Proestrus		Estrus		Metestrus		Diestrus I		Diestrus II	
	O	E	O	E	O	E	O	E	O	E
0	153	135.0	147	97.3	53	39.6	4	0.1	197	137.8
1	53	69.0	47	80.2	85	70.0	9	0.8	50	89.1
2	13	17.6	14	33.1	40	61.9	15	2.9	5	28.8
3	2	3.0	1	9.1	27	36.4	20	7.2	3	6.2
4	2	0.4	1	1.9	10	16.1	9	13.6		
5			4	0.31	7	5.7	22	20.6	1	0.13
6			2	0.04	3	1.7	14	25.8		
7			2	0.00	3	0.4	18	27.8	1	0.00
8			1	0.00	2	0.09	11	26.1	1	0.00
9	1	0.0					13	21.9		
10							6	16.5	1	0.00
11			1	0.00			8	11.3		
12			1	0.00			6	7.1		
13	1	0.0			1	0.00	6	4.1	1	0.00
14							8	2.2		
15							6	1.1		
16							6	0.5		
17							2	0.2	1	0.00
18							2	0.1	1	0.00
19							1	0.04	1	0.00
20							2	0.01		
21					1	0.00				
22										
23							2	0.00		
24			1	0.00						
Σ f	225	(225)	222	(221.9)	232	(231.9)	190	(189.9)	263	(263.0)

$Ne^{-\mu}$ = expected frequency when $r = 0$; $\frac{\mu^r}{r!}(Ne^{-\mu})$ = expected frequency when r = 1-24, N = sample size, e = base of natural logarithm, and $\mu = (\Sigma fr)/\Sigma f$.

Table 2—Goodness of Fit of Observed Frequency Distribution of Mitoses in Rat Mammary Gland Epithelium to Poisson Frequency Distribution (First Series)

Cycle phase	Rat no.	Fit to Poisson distributions		
		X^2	DF	*P*
Proestrus	1	6.36	3	NS
	2	2.95	3	NS
	3	23.03	4	<0.0005
	4	0.82	2	NS
	5	3.96	2	NS
	Pooled data	9.3	2	<0.01
Estrus	1	46.30	4	<0.0005
	2	45.75	5	<0.0005
	3	41.34	3	<0.0005
	4	6.32	3	NS
	5	0.80	2	NS
	6	0.41	2	NS
	Pooled data	108.8	3	<0.0005
Metestrus	1	6.34	4	NS
	2	20.25	5	<0.005
	3	6.27	5	NS
	4	10.56	6	<0.1
	5	28.16	5	<0.0005
	6	23.47	5	<0.0005
	Pooled data	48.4	5	<0.0005
Diestrus I	1	45.94	17	<0.0005
	2	78.80	18	<0.0005
	3	34.47	8	<0.0005
	4	44.89	12	<0.0005
	5	40.15	9	<0.0005
	Pooled data	335.5	11	<0.0005
Diestrus II	1	110.93	4	<0.0005
	2	26.92	3	<0.0005
	3	0.50	2	NS
	4	31.09	3	<0.0005
	5	0.23	2	NS
	6	0.39	2	NS
	Pooled data	65.5	2	<0.0005

X^2 = Chi-square value, $\sum (0 - E)^2/E$; DF = degrees of freedom (number of classes − 2; classes counted after making any combination of classes necessary because of small Poisson expectations); NS = not significant, $P > 0.05$. Pooled data from Table 1.

phases supported in part (50% of the rats in estrus, metestrus and diestrus II had significant Chi-squares) or completely (diestrus I rats) the deviation from randomness observed with the pooled data.

A second larger series of rats was studied to confirm the above results. Table 3 shows the observed and expected pooled frequency distributions of mitoses per unit volume of lobuloalveolar tissue for this series of rats (N = 79; 8 to 25 rats at each cycle phase). Again, the observed distributions deviated from the Poisson distributions at each phase of the estrous cycle. The deviations were, as in the first series, the result of an excess frequency

Table 3—Observed Frequency (O) and Expected Frequency (E) of Mitoses in Samples of Rat Mammary Gland Epithelium (Second Series)—Pooled Data

No. of mitoses/ unit volume = r	Cycle phase									
	Proestrus		Estrus		Metestrus		Diestrus I		Diestrus II	
	O	E	O	E	O	E	O	E	O	E
0	151	94.5	275	118.6	150	62.1	72	18.3	117	63.7
1	79	123.5	190	222.5	194	163.1	141	64.8	73	105.1
2	56	80.6	143	208.6	202	214.3	115	114.9	63	86.8
3	26	35.1	59	130.5	109	187.6	80	135.9	26	47.8
4	18	11.5	43	61.2	70	123.3	62	120.4	24	19.7
5	6	3.0	23	22.9	38	64.8	47	86.4	18	6.5
6	7	0.6	14	7.2	30	28.4	27	50.5	6	1.8
7	1	0.1	3	1.9	19	10.7	23	25.6		
8	1	0.02	3	0.45	9	3.5	9	11.3	1	0.09
9	2	0.00	2	0.09	6	1.0	8	4.5	1	0.02
10	1	0.00			12	0.37	10	1.6	1	0.00
11			2	0.02	5	0.06	13	0.51	2	0.00
12			1	0.00	2	0.01	5	0.15		
13			2	0.00	1	0.00	3	0.04		
14			1	0.00	2	0.00	3	0.01		
15			2	0.00	2	0.00	1	0.00		
16			2	0.00	1	0.00	3	0.00		
17					1	0.00	2	0.00		
18					3	0.00	2	0.00		
19			1	0.00			3	0.00		
20			1	0.00	1	0.00	2	0.00		
21	1	0.00					1	0.00		
22			2	0.00	1	0.00	1	0.00		
24										
26			1	0.00	1	0.00				
27			1	0.00						
28			1	0.00						
30							1	0.00		
31			2	0.00						
Σf	349	(348.9)	774	(773.9)	859	(858.9)	634	(633.9)	332	(331.5)

Refer to Table 1 for formulas for calculation of expected frequency.

of lobuloalveolar tissue with high densities of mitoses and an excess frequency of tissue without mitotic activity. Chi-square values for the pooled and individual rats are presented in Table 4. Chi-square values for the pooled data revealed that the deviations from random expectations were highly significant at each phase of the estrous cycle.

The conclusion of nonrandomness drawn for the pooled data was supported by the Chi-square values obtained for the majority of the individual rats which were significant in 67%, 75%, 88%, 47%, and 75% of the rats sampled at proestrus, estrus, metestrus, diestrus I, and diestrus II, respectively.

Considering the results of Series 1 and 2, these data indicate that 66.7% (72/108) of the individual observed mitotic frequency distributions deviated significantly from expected Poisson distributions.

Table 4—Goodness of Fit of Observed Frequency Distributions of Mitoses in Rat Mammary Gland Epithelium to Poisson Frequency Distribution (Second Series)

		Fit to Poisson distributions				Fit to Poisson distributions		
Cycle phase	Rat No.	χ^2	DF	*P*	Rat No.	χ^2	DF	*P*
Proestrus	1	14.3	3	<0.005	6	39.3	5	<0.0005
	2	7.8	3	<0.05	7	14.3	4	0.01
	3	89.2	8	<0.0005	8	0.7	3	NS
	4	15.1	3	<0.005	9	1.5	4	NS
	5	4.8	3	NS	Pooled data	116.5	4	<0.0005
Estrus	1	99.8	6	<0.0005	12	17.3	5	<0.005
	2	40.1	6	<0.0005	13	16.2	6	<0.025
	3	1.6	2	NS	14	17.7	6	<0.01
	4	9.0	3	<0.05	15	29.1	4	<0.0005
	5	6.2	4	NS	16	16.4	7	<0.025
	6	7.8	4	<0.1	17	9.9	5	<0.1
	7	63.5	5	<0.0005	18	51.6	5	<0.0005
	8	474.9	8	<0.0005	19	28.8	6	<0.0005
	9	42.9	7	<0.0005	20	6.1	2	<0.02
	10	4.3	3	NS	Pooled data	524.2	6	<0.0005
	11	31.8	6	<0.0005				
Metestrus	1	30.9	6	<0.0005	14	18.4	9	<0.05
	2	10.0	5	<0.1	15	3.2	5	NS
	3	10.1	6	NS	16	2.7	5	NS
	4	7.1	4	NS	17	18.5	6	<0.01
	5	10.3	4	<0.1	18	15.4	5	<0.01
	6	48.6	6	<0.0005	19	86.3	8	<0.0005
	7	35.1	4	<0.0005	20	46.4	9	<0.0005
	8	28.8	6	<0.0005	21	18.5	6	<0.01
	9	32.7	5	<0.0005	22	15.4	5	<0.01
	10	22.8	6	<0.001	23	53.8	10	<0.0005
	11	24.4	8	<0.005	24	88.0	8	<0.0005
	12	2.1	4	NS	25	103.9	10	<0.0005
	13	1.0	4	NS	Pooled data	48.4	5	<0.0005
Diestrus I	1	4.3	6	NS	10	45.9	9	<0.0005
	2	3.8	7	NS	11	24.9	10	<0.01
	3	17.6	5	<0.0005	12	102.2	13	<0.0005
	4	5.7	5	NS	13	14.1	9	NS
	5	6.3	4	NS	14	68.5	7	<0.0005
	6	2.2	5	NS	15	5.3	6	NS
	7	1.3	3	NS	16	10.7	6	<0.1
	8	222.3	16	<0.0005	17	47.2	7	<0.0005
	9	42.6	12	<0.0005	Pooled data	335.5	9	<0.0005
Diestrus II	1	25.4	4	<0.0005				
	2	19.6	5	<0.0005				
	3	114.8	3	<0.0005				
	4	10.3	5	<0.1				
	5	10.8	5	<0.1				
	6	10.9	4	<0.05				
	7	13.7	4	<0.01				
	8	23.3	4	<0.0005				
	Pooled data	65.5	2	<0.0005				

X^2 = Chi-square value, $\sum(O - E)^2/E$; DF = degrees of freedom (No. of classes − 2; classes counted after making any combination of classes necessary because of small Poisson expectations). NS = not significant, $P > 0.05$. Pooled data are from Table 3.

The mean mitotic activity of lobuloalveolar mammary epithelium at different phases of the estrus cycle for the two experimental series are shown in Table 5. In both series, mean mitotic activity rose from proestrus to reach a maximum in diestrus I and then fell in diestrus II, thus providing a qualitatively similar cyclical variation in mitotic activity during the estrus cycle. Note, however, that in the second series, mean mitotic activity at each phase of the cycle other than diestrus I was higher than in the first experimental series. This may explain, in part, the higher incidence of rats in the second series which had nonrandom mitotic frequency distributions.

Discussion

Our results indicate that the frequency distribution of mitoses per unit volume of lobuloalveolar mammary epithelium deviates significantly from Poisson distributions. The deviations from randomness were related to the following: a) the frequency of lobuloalveolar tissues with no mitoses exceeded the predicted Poisson frequency distribution and b) the frequency of lobuloalveolar tissues with high densities of mitoses exceeded the frequency predicted by the Poisson distribution.

At least two possible explanations which may not be mutually exclusive are suggested by our results: a) mitoses may, in part, be initiated by a local increase or decrease of a cell product, i.e., mitotic cells may stimulate the division of other cells in their immediate vicinity, and b) a relatively high degree of cellular replicative synchrony may exist in individual lobuloalveolar tissue units such that the cells of these units tend to undergo mitosis in unison.

The first possibility has previously been suggested by other investigators to account for mitotic clustering. For example, Rowe and Dixon [8] studied the distribution of mitoses in biopsies of human epi-

Table 5—Mean Mitotic Activity of Rat Lobuloalveolar Mammary Epithelium at Different Phases of the Estrous Cycle

Cycle phase	No. of mitoses/cu mm (mean ± SE)	
	Series 1*	Series 2
Proestrus	4,500 ± 800	9,000 ± 1,000
Estrus	6,400 ± 1,100	11,000 ± 1,000‡
Metestrus	12,000 ± 900†	17,000 ± 1,000‡
Diestrus I	57,200 ± 3,300†	21,000 ± 1,000‡
Diestrus II	3,100 ± 600†	16,000 ± 1,000‡

* Data from Purnell and Kopen.[11]
† Comparisons between phases are significant, $P < 0.01$ or less, Student's t test, two-ended.
‡ Comparisons between phases are significant, $P < 0.005$, Student's t test, two-ended.

dermis. In their study, they enumerated mitoses in a defined area of tissue and compared these to expected Poisson distributions. Mitoses in contact with each other were excluded on the assumption that they might be sister cells of a previous mitosis, which would tend to divide synchronously. In spite of this correction, they found an excess frequency of mitotic clusters (two or more mitoses/unit area of tissue) over chance expectations.

Direct evidence for a spreading mitotic stimulus was reported by Cone.[14] Using an *in vitro* system of neoplastic mouse fibroblasts, he obtained time-lapse cinephotographic data which indicated that a rapid mitotic stimulus was spread sequentially from one dividing cell to neighboring cells via intercellular connections. This demonstrated that groups of interconnected cells could become mitotically autosynchronized. Whether this phenomenon occurs under *in vivo* conditions is not known, but intercellular cytoplasmic connections have been implicated in the synchronous *in vivo* differentiation of spermatids into mature sperm.[15]

If a spreading mitotic stimulus were operative in our system, it would explain the excess frequency of lobuloalveolar tissue with high densities of mitoses, but would not account for the similar excess of mitotically inactive areas.

No direct evidence is available to support the alternative possibility (replicative synchrony). Nevertheless, Bresciani [16] previously suggested that a high degree of alveolar cell replicative synchrony might underlie the variation in the proportion of DNA-synthesizing cells which he observed between individual alveolar units of the mouse mammary gland. Such a phenomenon could also explain our observations. The excess observed frequency of lobuloalveolar tissues without mitoses as well as those with a high density of mitoses could represent tissue units composed of cells having a high degree of replicative synchrony.

In conclusion: The results of our study demonstrate a nonrandom pattern of mitotic activity in the lobuloalveolar tissue of the rat mammary gland. These data suggest that local cell products, e.g., mitotic stimulators or a variation in the replicative synchrony of lobuloalveolar cell populations may determine in part the pattern of mitotic activity in the rat mammary gland. Our data may have implications for mammary gland tumorigenesis. For example, a nonrandom distribution of mitotic activity could account in part for the focal nature of mammary neoplasia, i.e., lobuloalveolar tissue units with high replicative activity may be at the highest risk for neoplastic transformation following exposure to a carcinogenic stimulus. In support of this, Nagasawa *et al.*[17-18] showed earlier that the yield of rat mammary tumors is directly related to the extent of mammary gland DNA synthesis at the time of carcinogen administration.

Finally, lobuloalveolar tissues with high mitotic activity may be more likely to develop into premalignant hyperplastic lesions than tissues having lower mitotic activity if the high level of mitotic activity in such tissues continues over a period of several estrous cycles. Studies of the distribution of mitotic activity in the mammary glands of rats differing in their incidence of spontaneous or induced hyperplastic alveolar nodules and mammary tumors might shed some light on these questions.

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