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Preferential Accumulation of Iron in Hyperplastic Tissue of Rat Mammary Gland (41250)

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Abstract. Quantitative measurements of the iron contents of various rat mammary tissues (normal, hyperplastic, and tumor) revealed that hyperplastic tissues contained significantly more iron than normal and tumor tissues. Normal mammary glands from virgin, pregnant, lactating, and retired breeder rats had an iron content ranging from 21 to 73 μg/100 mg dry weight ($\bar{X}$ = 43), while that of primary and transplantable DMBA-induced mammary tumors ranged from 31 to 52 μg/100 mg dry weight ($\bar{X}$ = 38). In contrast, the iron content of hyperplastic mammary tissue, both DMBA-induced and spontaneous, was 139 to 204 μg/100 mg dry weight ($\bar{X}$ = 176). Thus, in addition to their previously reported growth alterations, rat mammary hyperplasias are also physiologically altered from normal in their sequestering of iron. Although the mechanisms involved are unknown, this property should provide a useful quantitative index for *in vivo* assays of mammary hyperplasias induced *in vitro*.

A striking characteristic of hyperplastic alveolar nodules is their yellow–orange color in the mammary glands of rats treated with the chemical carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) (1) and of mice infected with mammary tumor virus (MTV) (2). Indeed, because of their coloration, these lesions are readily detectable in fresh, unstained mammary preparations as discrete orange lobules against a pale cream background. Histochemical staining indicates that the orange color is due to iron deposits in the nodules (4), presumably in the form of hemosiderin (2, 5). The presence of hyperplastic nodules in the mammary glands of (mammary) tumor-bearing mice is said to account for the observation that their stainable iron content is higher than in glands of non-tumor-bearing mice (6). However, it should be noted that stainable iron deposits are also demonstrable in normal mammary glands (4–7), whereas mouse mammary tumors appear to be devoid of stainable iron (8, 9). Because these previous studies have relied mainly on histochemical evidence for iron, it was of interest to determine whether chemical analysis would reveal quantitative differences in the iron content of various mammary tissues. In addition, since hyperplastic mammary nodules are altered from normal in their growth and developmental properties (10–12), it was reasonable to suspect physiological alterations as well, such as might be reflected in their content of iron. Should iron accumulate selectively in mammary nodules, this property might provide a useful index for the *in vivo* evaluation of mammary hyperplasias induced *in vitro*. Accordingly, the present study was undertaken using the rat mammary gland system to determine whether hyperplastic alveolar nodules accumulate more iron than do tumors or normal mammary tissues.

Materials and Methods. *Animals.* Two sublines of Lewis rats, separately maintained and inbred by brother–sister matings in our laboratory since 1976, provided the mammary tissues for this study. Most of the tissues were from females of strain LEW/SsN received from the National Institutes of Health, Bethesda, Maryland, the remainder from strain LEW/Sim received from Simonsen's Laboratories, Gilroy, California. Animals were provided Lab-Blox pellets (Wayne Laboratory Animal Diets, Chicago, Ill.) and tap water *ad libitum* under a 12-hr light–dark cycle.

Induction of hyperplastic alveolar nodules and mammary tumors. Virgin females were fed 10 mg DMBA (Sigma Chemicals, St. Louis, Mo.) in 1 ml of sesame oil by gastric intubation, once at 45–50 days of age and again 1 week later (total dose, 20 mg per rat). A high incidence of hyperplastic alveolar nodules and mam-

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mary tumors develop within 3–4 months after DMBA feeding.

Hyperplastic nodule outgrowths. The microscopic size of hyperplastic nodules (1–2 mm in diameter) precluded chemical analysis of their iron content. To overcome this problem, hyperplastic mammary outgrowths were studied. These were prepared by transplanting nodules singly into gland-free inguinal mammary fat pads of syngeneic hosts as described before (13) and modified for the rat by Rivera *et al.* (12). By 10–12 weeks, nodules proliferate to fill most of the fat pads with hyperplastic outgrowth. These outgrowths were individually analyzed after removal of extraneous fat and connective tissue. The first generation growths in the fat pads are designated *primary nodule outgrowths*. Those transplanted serially in mammary fat pads are designated *nodule outgrowth lines* (E. M. Rivera, manuscript in preparation). With the exception of one nodule outgrowth line, T43, derived from a hyperplastic nodule which arose spontaneously in an untreated old female, all primary and transplantable hyperplasias were derived from DMBA-induced hyperplastic alveolar nodules.

Mammary tumors. Mammary tumors, approximately 1.5–2.0 cm in diameter, were analyzed individually for their iron content following removal of the connective tissue capsule and if present, necrotic portions of the tumor mass. *Primary mammary tumors* were those selected randomly from the mammary glands of DMBA-treated rats. DMBA-induced *mammary tumor lines* were those established in our laboratory by serial transplantation in gland-free mammary fat pads (E. M. Rivera, manuscript in preparation).

Normal mammary tissues. Mammary glands were dissected from untreated adult females in various stages of physiological development as indicated in Table I. These

TABLE I. IRON CONTENT OF VARIOUS RAT MAMMARY TISSUES

Tissue[a]	Iron[b]	
	μg/100 mg dry weight	μg/mg protein
A		
Primary nodule outgrowths	204.0 ± 28.3[c] (3)	
Nodule outgrowth line (T43)	184.0 ± 41.2[d] (4)	
Mammary tumor line (T41)	31.3 ± 5.1 (4)	
Mammary glands, virgin females[e]	53.3 ± 13.4 (4)	
Liver	166.8 ± 3.7 (4)	
Spleen	217.5 ± 23.2 (4)	
B		
Primary nodule outgrowths	138.7 ± 10.2[f] (7)	3.70 ± 0.42[f] (7)
Nodule outgrowth line (T19, T30)	177.1 ± 13.4[f] (8)	5.23 ± 0.80[f] (5)
Primary mammary tumors	51.8 ± 1.4 (4)	0.91 ± 0.06 (4)
Mammary tumor lines (T47, T52)	30.7 ± 4.5 (4)	0.39 ± 0.10 (4)
Mammary glands, virgin females[g]	23.5 ± 1.3 (5)	0.81 ± 0.04 (5)
Mammary glands, pregnant females	41.6 ± 7.7 (6)	0.99 ± 0.12 (6)
Mammary glands, lactating females	21.3 ± 0.02 (2)	0.33 ± 0.01 (2)
Mammary glands, retired breeders	73.4 ± 6.9 (14)	1.42 ± 0.14 (14)
Liver	200.0 ± 11.0 (2)	1.83 ± 0.10 (2)

[a] Transplantable nodule and tumor lines are identified in parentheses.

[b] Values are the means ± SE for the numbers of individual samples shown in parentheses.

[c] Value significantly greater than that of mammary tumor line (T41) ($P < 0.001$) and mammary glands, virgin females ($P < 0.01$).

[d] Value significantly greater than that of mammary tumor line (T41) ($P < 0.02$) and mammary glands, virgin females ($P < 0.05$).

[e] Females were 6–13 months old.

[f] Values significantly greater than all tumor and normal mammary gland groups in B ($P < 0.001$).

[g] Females were 2 years old.

included young and old virgin females, breeding females, and retired breeders. Each gland was individually analyzed after removal of lymph nodes and extraneous connective tissue.

Iron estimation. Tissues were defatted overnight in acetone, 200 ml/g of tissue, and then dried to constant weight at 110°C. For the earlier part of the study (Table IA), iron was extracted by the wet-ashing technique, in which dried tissues were digested in a perchloric–nitric acid mixture (14). The tissue digest was then made up to 10 ml in volumetric flasks, and 1-ml aliquots were taken for iron estimation by the *o*-phenanthroline method (14). An alternate procedure (15) for the extraction of nonheme iron was used for the remainder of the study (Table IB), since it permitted determination of total protein content of the tissues. In this case, the defatted–dehydrated tissues were homogenized in 2x-distilled water and made up to 50 ml in the presence of 20 ml of 10% hydrochloric acid. The homogenate was heated at 80°C for 15 min and centrifuged at 1500g. Protein was estimated by the method of Lowry *et al.* (16) on aliquots of the supernatant and NaOH-solubilized precipitate. Two milliliters of 40% trichloroacetic acid were added to 10-ml aliquots of the remaining supernatant and left for an additional 10 min at 80°C. After centrifugation at 300*g* for 20 min, 1-ml aliquots of the protein-free supernatant were taken for iron estimation by the *o*-phenanthroline method (14).

o-Phenanthroline, iron and protein standards, and Folin's reagent were purchased from Sigma Chemicals.

Liver and spleen from virgin female rats were taken as positive control tissues for the estimation of iron.

Results and Discussion. The results in Table I show that there are significant differences in the amounts of iron present in the various types of rat mammary tissues. Expressed on a dry weight basis, the iron content of hyperplastic mammary tissue was two- to eight-fold higher than that of normal tissue and three- to seven-fold higher than that of mammary tumors. When the iron values are expressed per milligram protein, hyperplasias contain 4 to 13 times more iron than normal and 3 to 16 times more iron than tumor tissues. Because of the range of iron values obtained for the different sources of tissue, it is also useful to compare their mean values. Thus, when the average iron contents (176, 38, and 43 μg/100 mg dry weight of hyperplasias, tumors, and normal mammary tissue, respectively) are compared, it is still evident that hyperplastic tissues accumulate 4 to 5 times more iron than normal and tumor. These quantitative data show clearly that iron accumulates preferentially in DMBA-induced and spontaneous hyperplasias of rat mammary gland and furthermore, their iron content is comparable to or greater than that of liver and spleen, the normal storage sites of iron.

The results are also noteworthy because they are a striking contrast to observations in rat liver, where hyperplastic nodules and tumors induced by chemical carcinogens are *resistant* to iron accumulation when the liver is rendered siderotic by iron overload (17). The reasons for this difference in behavior of hyperplastic nodules of liver and mammary gland are not apparent but may be related to the fact that the liver normally functions as a storage site for iron whereas the mammary gland does not. It is possible that the alterations induced by chemical carcinogens depend upon the cell types affected.

The significance of iron accumulation in mammary hyperplasias is unknown, and in fact, little is clearly understood of the trapping of iron in milk and mammary tissue or of the transport mechanisms involved in iron distribution (18, 19). Nevertheless, there is increasing evidence that alterations in iron metabolism and ferritin are associated with breast and other cancers (20–23). Women with early breast cancer have serum ferritin levels higher than those of normal control patients (20), and although this increase in serum ferritin may not be specific to malignancy, it does indicate that iron metabolism is altered in breast cancer patients.

Rawlinson and Pierce (24) reported some years ago that human breast biopsy mate-

rial diagnosed as adenosis showed areas of stainable iron deposits in the alveoli and ducts, as did a human mammary hyperplasia (H. E. Rawlinson, unpublished, cited in ref. (7)). As far as we are aware, there have been no recent studies on the iron content of human mammary hyperplasias.

The earlier observation that mouse mammary tumors were devoid of stainable iron (8, 9) is at variance with the chemical results reported here from rat mammary tumors, although the amounts of iron in the tumors were low compared to that of hyperplasias. A similar discrepancy was reported for lactating rat mammary gland, which contains chemically detectable but not stainable iron (18). In our laboratory, stainable iron was demonstrable in normal and hyperplastic mammary tissue but not in mammary tumors. It is possible that histochemical methods detect only limited forms of bound iron, probably that associated with hemosiderin.

Because most of the intracellular nonheme iron is bound to the proteins ferritin and hemosiderin (21), we plan to examine the question of whether iron is differentially bound in the various mammary tissues of the rat (normal, hyperplastic, and tumor). In addition, it will be of interest to determine whether mammary hyperplasias induced by chemical carcinogens *in vitro* and transplanted *in vivo* (25) show also a preferential accumulation of iron compared to normal mammary tissues. Studies similar to those reported earlier (25) are in progress in our laboratory. Whatever the explanation for the sequestering of iron in mammary hyperplasia, it opens up for further investigation its relationship to the growth and oncogenic potential of these lesions.

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