Cell types and morphogenesis in the mammary gland

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ABSTRACT Cell types present in the mammary gland and their evolution were studied by labeling female rats with radioactive thymidine at various phases of the estrus cycle. The results suggest that the stem cells for mammary development are present in the terminal end buds and that they generate a lineage for luminal cells and possibly a distinct one for myoepithelial cells. Growth and differentiation are controlled by both hormones and local factors.

The mammary gland is the site of very frequent cancers in humans as well as in animals. The formation of these cancers appears to be influenced by the developmental changes occurring in the gland after birth: in female rats the inducibility of cancer by chemicals is maximal at 50–60 days of age, when the changes are at their peak; pregnancy, lactation, and hormone treatments affect the incidence of mammary cancer in women and in animals. Therefore, a knowledge of the cell types present in the mammary gland and their evolution may be useful for understanding carcinogenesis.

In the young rat the mammary gland consists of a system of branching ducts, which terminate with actively growing structures, the terminal end buds (TEB); the ducts show branching (sometimes extensive) into ductules, also called alveolar buds. Essentially only two cell types are clearly recognized in these structures: the epithelial cells that line the lumen of ducts and ductules and the myoepithelial cells that surround them. However, three types are recognized from nuclear morphology (1). In lactation the alveoli (which derive from ducts and ductules) are lined by functionally different cells, those producing milk, which also are surrounded by myoepithelial cells. The differentiation of the mammary gland is under the control of multiple hormones. Many observations show that estrogens promote end-bud development and duct elongation and that progesterone promotes duct enlargement and ductule formation and growth (2–7).

We have taken advantage of the hormonal changes occurring during the normal estrus cycle of the mature virgin female rats to identify cell types with differential growth responses. The basic approach was to label the cells by an injection of [3H]dThd into the animal at defined phases of the estrus cycle and to detect the DNA-synthesizing cells in histological slices by autoradiography (4–10). By allowing time periods of various lengths between injection and removal of the gland for examination, pulse-chase experiments also were performed. These approaches identify several kinds of epithelial mammary cells and suggest a possible developmental pathway. The experiments were complemented by studies with various cell markers, which will be reported separately.

MATERIALS AND METHODS

Forty-two female Sprague–Dawley rats were used, most as cycling adults (50–60 days of age) and some as immature animals (ca. 25 days). The estrus phases were determined by vaginal smears. Groups of animals were injected intraperitoneally with [3H]dT (20 Ci/mmole; 1 Ci = 3.7 × 1010 becquerels), by using 0.5 mCi for adults and 0.25 mCi for immature animals. Animals then were killed 1, 24, or 48 hr later.

The abdominal-inguinal mammary glands were dissected and fixed in buffered formalin. Stained whole mounts were prepared. Fragments rich in end buds, in ducts, or in ductules were isolated under a dissecting microscope; they were embedded in paraffin. Serial sections, 8-μm thick, were mounted on glass slides. The hydrated slides were covered with liquid photographic emulsion (Ilford or Kodak) diluted with 2 vol of water. The films were exposed for 7–10 days; they then were developed, fixed, and stained with hematoxylin/eosin and, in addition, were stained with periodic acid and a Shiff base reagent for identification of the basement membrane. They were mounted in Permount. For observation of nuclear morphology, films were exposed for a shorter time, as needed, in order to decrease the number of grains. For tridimensional reconstructions, appropriate areas of each section were photographed and reproduced on glass slides, which then were glued together with Permount in the proper sequence and alignment. Photographic grains were counted under oil immersion, background was ca. 0.25 grain per nuclear area. Above ca. 50 grains per nucleus the numbers had to be estimated, but comparisons of radioautographs exposed for different times showed that these estimates are sufficiently correct. The results are not affected by the geometry of the nuclei within the sections because they are based on comparisons of labeling indexes (LIs) or grain counts rather than on absolute numbers.

RESULTS

Effect of [3H]dT injection. Radioactivity was determined in blood samples withdrawn at subsequent intervals from rats injected with [3H]dT. Considerable radioactivity persisted for many hours, but paper chromatography showed that most of it was not in dT. The persistence of labeled precursors for DNA synthesis was determined by adding aliquots of serum to subconfluent cultures of RAM 25 cells (derived from a rat mammary carcinoma induced by 7,12-di methylbenz[a]anthracene) (11). Analysis of incorporation of label into cellular DNA showed that precursors of DNA synthesis were present in the serum during the first hour after injection but were absent after 2 hr. Therefore, the [3H]dT injection corresponds to a pulse of precursor lasting between 1 and 2 hr.

Abbreviations: LI, labeling index; TEB, terminal end buds.

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Distribution of Label After a 1-Hr Pulse of \(^{3}H\)dThd. Thirty animals were used. Reconstructions of fragments of the mammary gland injected with \(^{3}H\)dThd at various phases of the estrus cycle identify unambiguously several labeled structures: the terminal end buds, the ducts, and the ductules (Fig. 1). The differences in labeling were investigated by determining LI for various cell types as a function of the estrus cycle at the time of injection. The data are reported in Figs. 2 and 7.

End Buds. To eliminate variability deriving from differences of LI among different parts of the same bud, whole end buds were studied, by using serial sections. The LI was found to be high (between 10% and 40%) in both immature and cycling animals (Table 1). In addition, cycling animals showed two peaks, one at early estrus and the other at late estrus–metestrus (Fig. 2). At all ages the labeled cells formed groups separated by

Table 1. Labeling characteristics at various times after \(^{3}H\)dThd injection

<table>
<thead>
<tr>
<th>Animals</th>
<th>Estrus cycle</th>
<th>Time, hr(^*)</th>
<th>LI</th>
<th>Grains(^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature</td>
<td>1</td>
<td>0.14</td>
<td>88.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.54</td>
<td>36.8</td>
<td></td>
</tr>
<tr>
<td>Mature proestrus</td>
<td>1</td>
<td>0.17</td>
<td>39.8(^*)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.12</td>
<td>29.8(^*)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.43</td>
<td>20.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.56</td>
<td>26.0</td>
<td></td>
</tr>
<tr>
<td>Early estrus</td>
<td>1</td>
<td>0.38</td>
<td>80.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.27</td>
<td>69.8</td>
<td></td>
</tr>
</tbody>
</table>

One experiment is shown per line.

\(^*\)Time at which animals were killed.

\(^*\)Per radioactive nucleus.

In these animals, grain counts over all types of cells were low, showing a less efficient labeling.

groups of unlabeled cells, suggesting that local subpopulations are engaged in growth.

In cycling animals at proestrus, labeled cells, most with large oval dark nuclei, are concentrated around the periphery of the end bud (Fig. 3); in subsequent phases, labeled cells with large round pale nuclei appear throughout the bud, until at metestrus they are the main labeled type and are concentrated around the lumen. At the same time the buds change in size and shape. They increase in size from proestrus to late estrus, maintaining a smooth outline. At metestrus they become smaller and have indentations. They are smallest at diestrus. These results suggest that there is a regular phase-dependent evolution of cell types within the end buds (Fig. 4).

The stroma surrounding the buds and separating them from the adipose cells of the fat pad always contained many labeled
cells; the LI was comparable to that of the bud epithelium itself. In contrast, labeled cells in the fat pad far from the bud were rare. During proestrus and early estrus the border between the tips of the end buds and the surrounding stroma is indistinct and is straddled by replicating cells. Locally the stroma appears very loose (Fig. 5).

Ducts. In large ducts—i.e., with more than 100 cells in a cross section—the labeling of myoepithelial cells could be studied unambiguously in tangential sections in which the myoepithelial cell nuclei are longitudinally arranged (Fig. 6). In all other sections the myoepithelial cell nuclei could not be identified unambiguously. Therefore, we have included the cells at the outer surface of ducts within the basement membrane in a single class as "outer cells," they are mainly myoepithelial cells but may include some peripheral epithelial nuclei. Cells close to the lumen were kept separate as "inner cells."

The data given in Fig. 7A show that the LI depends strongly upon the phase of the cycle for both cell types. Outer cells have a higher LI, especially at diestrus and proestrus. Myoepithelial cells, when clearly identified in tangential sections, have the highest LI of all ductal cells.

Ductules. Ductules are short branching trees deriving from ducts. The LIs of ductules observed in random slices of central parts of mammary glands show considerable variation. Serial sections show that there are differences both between ductular trees and within a tree; this is because peripheral parts of ductules bordering on the free fat pad tend to have higher LIs, sometimes markedly so. Parts with different LIs are morpho-

Fig. 5. Tip of TEB at proestrus. The TEB extends to the left. (×330.)

Fig. 6. Tangential section of duct showing elongated myoepithelial cells with high LI. (×370.)

logically similar; therefore, the heterogeneity appears to depend upon local factors. We have compensated for this heterogeneity in two ways: in some cases we determined the LI in complete ductular trees; in other cases the LI was determined in all ductular sections present in many tissue slices, irrespective of topography. In both cases the cross sections were grouped in classes according to the total number of nuclei they contained. Within each class the mean and the variance of the distribution of labeled cells per cross section were determined. Excess of variance over mean indicates heterogeneity.

The data (Fig. 8) show that heterogeneity is low in cross sections with up to 30 cells, but it increases in larger cross sections, in which the LI also is higher. Examination of serial sections shows that the cross sections correspond to ductules with dilated ends that reach furthest into the fat pad. The class of the smallest cross sections often had a higher heterogeneity because it in-
Fig. 8. LI of ductule cross sections of different sizes in representative animals. Cells, number of cells per cross section. P, proestrus; D, diestrus; E, early estrus; M, middle estrus; L, late estrus; MET, metestrus. Values are means ± 2 SD.

includes cross sections of the very tips of the dilated ends. The overall LIs of ductule cross sections with less than 40 cells, which are the most uniform, are given in Fig. 7B.

**Distribution of \(^{3}\)HdTd Label 24–48 Hr After Injection.** Twelve animals were labeled at various phases of the cycle.

End Buds. In both immature and cycling animals the LI was higher, up to 4-fold after the chase, than in glands examined 1 hr after injection and the average number of grains per nucleus was correspondingly lower (Table 1). However, the total number of grains per section was approximately the same. These changes suggest that a sizable proportion of the labeled cells divided twice as average during the chase period. The increase in LI implies that during the chase multiplication occurred preferentially in the cells that were labeled during the first hr after injection. This shows that the patches of replicating cells detected after 1 hr continue to grow during the chase with little recruitment of new cells. These cells represent the growing compartment of the end buds.

In rats injected in proestrus or early estrus, nuclei with the highest number of grains were found regularly near the lumen of the buds after a 48-hr chase (Fig. 9). This distribution contrasts with predominance of labeled nuclei in the outer layer of the buds after 1 hr at proestrus. These observations suggest that some of the cells labeled in proestrus or early estrus stop replication and migrate to the inner layer.

Ducts. In the ducts of glands examined 48 hr after an injection in late estrus (when the LI in the ducts is highest), the overall LI was increased after the chase because the labeled cells underwent division. More inner cells than outer cells were labeled, suggesting that some of the outer cells (of unknown origin) that were labeled at 1 hr moved to the lumen. Pairs of adjacent labeled cells with comparable numbers of grains were observed very frequently at the lumen, showing that the daughter cells did not move after division. No similar pairs of an outer and an inner cell could be found.

**DISCUSSION**

A single intraperitoneal injection of \(^{3}\)HdTd was given to either immature (25 days) or young (60 days) mature virgin female rats at various phases of the estrus cycle. The injection acts as a pulse of radioactive precursor lasting between 1 and 2 hr. The label persists in the mammary glands for at least 48 hr: during this time the pattern of cell multiplication and displacement can be followed.

The different growth response to the phases of the cycle permits the identification of several distinct cell types. At least two types of cells can be identified in the end buds: cells with oval dark nuclei, which make DNA preferentially at proestrus–early estrus, and another type with round pale nuclei, which makes DNA preferentially during late estrus–metestrus. Epithelial cells of ducts and ductules synthesize DNA, especially during middle and late estrus–metestrus; however, the response of the cells of ductules is displaced toward metestrus, suggesting that the control of the cells of ductules is different from that of the cells of ducts. In both ducts and ductules, myoepithelial cells follow the phasing of the epithelial cells but show a somewhat higher LI.

There is a characteristic evolution of cell types in the end bud, which is revealed by changes in shape, size, and LI. At diestrus, when the end buds are smallest, they have the lowest LI. At proestrus the buds are longer and show active DNA replication in cells with oval dark nuclei of the outer layers, especially at the tips. Myoepithelial cells cannot be identified in this area, and cells straddle the boundary between the bud and the stroma. These observations suggest that beginning at proestrus, the buds extend into the fat pad by multiplication of the cells with oval dark nuclei at their tips and perhaps also by cell migration into the stroma. During estrus the buds are longer and the labeled cells are distributed at random throughout the bud and include many cells with large pale round nuclei. In metestrus the buds are smaller and indented; many labeled cells with large pale round nuclei are found along the lumen of the bud stem. This part is now more similar to a duct, having well-oriented myoepithelial cells.

The results of the chase experiment suggest that the large pale cells derive from the migration and differentiation of cells
from the tips, which multiply at proestrus. These cells are similar in morphology to cells of ducts; they may be precursors of luminal ductal cells. The myoepithelial cells first appear in an area of the bud in which the cells with large oval nuclei are abundant and may derive directly from them. Shrinkage of the buds in metestrus and diestrus can be attributed to the migration of cells out of the buds to form a segment of duct. As they differentiate and reach the lumen, the cells retain their high replication rate for awhile, after having changed morphology. At diestrus the former bud stem has become a duct.

These observations suggest that the cells with oval dark nuclei at the tips of ducts are the stem cells for development of the mammary gland: they generate the lineage of the inner ductal cells. They also may generate the lineage of myoepithelial cells, but the evidence is not conclusive. The stem cells are responsible for the elongation of ducts into the fat pad. Ductules begin producing ductules both in the most actively replicating cells in the ducts, which also may act as precursors of alveoli during pregnancy. In the ducts, epithelial and myoepithelial cells appear to form two independent compartments. The myoepithelial cells are among the most actively replicating cells in the whole gland and may play important roles in growth and morphogenesis within the gland. When ducts begin producing ductules both myoepithelial and epithelial cells show a high LI. The contractile function of myoepithelial cells probably becomes developed during lactation; in fact, the alveolar myoepithelial cells are quite different in morphology from those lining the ducts (unpublished results).

The differentiation of the mammary gland is driven by a combination of hormonal and local controls. The multiplication of the cells with oval dark nuclei at the tip of buds, which is initiated in proestrus, before ovulation, probably is promoted by estrogen, together with other nonovarian hormones and local factors. The differentiation of stem cells into those with large pale nuclei, their migration toward the lumen, and their subsequent multiplication in middle to late estrus and metestrus, probably are promoted by progesterone in conjunction with nonovarian hormones. The multiplication of ductal and ductule cells in middle to late estrus and metestrus probably are also progesterone dependent. These conclusions are in agreement with previous observations. Local factors, probably produced by cells of the fat pad, may be responsible for the high LI at the periphery of ductules. The well-known observation that mammary gland cells only organize and grow when transplanted to the mammary fat pad suggests that these factors are specific.

Especially interesting is the stromal layer surrounding the end buds; it is very rich in cells, which display a high LI. At the very tip of buds the stroma is very loose and its limits with the epithelium are indistinct. These findings suggest interactions between stroma and epithelium. The growing ducts may release factors that stimulate mesenchyme growth and angiogenesis, whereas factors from the mesenchyme promote the growth of the end bud. Perhaps an end bud grows in an area softened by proteolytic digestion of collagen fibers.

The properties of the stem cells may be relevant for the behavior of the cancers experimentally induced or spontaneously arising in the mammary gland. The participation of end buds in carcinogenesis in rats induced by 7,12-dimethylbenz[a]anthracene (12) supports this view. The variable hormone dependence of various cancers also may be related to that of the developmental stage represented in the cancer. The effect of pregnancy on cancer incidence also may depend upon its effect on mammary development. We hope that the results presented in this paper will be useful for an experimental attack of these problems.

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