Marker Evolution during the Development of the Rat Mammary Gland: Stem Cells Identified by Markers and the Role of Myoepithelial Cells

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ABSTRACT

Using monoclonal antibodies and other immunological reagents we have identified characteristic markers for various epithelial cell types within the rat mammary gland. We have followed the evolution of cell types from the emergence of mammary ducts from the epidermis in the fetus to adulthood. Throughout mammary development some cells retain a group of markers which characterize the early stages of development. We have previously suggested that these cells are the stem cells for mammary development. In the adult, these cells are present in end buds and in the myoepithelial layer of ducts. We suggest that the myoepithelial layer, which we propose should be called the "basal" layer, contains several cell types, of which two are pluripotent. It contains the stem cells for mammary development, which also are present in end buds, and a precursor of ductules and alveoli. In the ducts, basal cells are probably also the precursor of luminal cells. We propose a scheme of mammary development.

INTRODUCTION

A study of the cell types involved in mammary carcinogenesis requires a precise description of the types present in the normal mammary gland. In order to evaluate the significance of the cell types present in cancers, the developmental evolution of the normal cell types is also important. We report here studies dealing with these two questions in the rat mammary gland using molecular markers for characterizing cell types. This will serve as background for an extensive characterization of rat mammary tumors induced by N-nitrosoethyurea, which has been carried out in this laboratory. Mammary glands originate from epidermal epithelium, forming ducts that penetrate into the fat pad where they ramify. In the young virgin female rat (3 wk of age) the ducts are made up of two layers of epithelial cells, an outer myoepithelial layer and an inner luminal layer. The ducts are terminated by semi-solid end buds. We have previously characterized the cells present in the 3- or 7-wk virgin glands using immunological markers, either as monoclonal antibodies to cultured cells derived from a rat mammary carcinoma or as sera to purified proteins (1, 2). We have identified several different cell types and ordered them in a developmental pathway. The ordering was based on the postulate that cells sharing the same markers are developmentally connected in a direct way. Based on these observations we have tentatively identified the putative stem cells for mammary development as a class of cells present in end buds. All the other cell types can be arranged in two lineages, luminal and myoepithelial, connected to these putative stem cells. In our previous work we found no evidence for connections between the two lineages.

In order to test whether stem cells are present exclusively in end buds, we have carried out experiments in which fragments of mammary glands not containing end buds were grafted to parenchyma-free mammary fat pads. The results showed that stem cells for mammary development are also present outside the end buds, in agreement with previous work (3); they moreover suggest the existence of a second type of pluripotent cell located in ducts which give rise to ductules and, in pregnancy, alveoli.

In the present work we have extended the studies of marker distribution to include earlier stages of mammary development, both prenatal and postnatal. We have also searched for cells with the markers of putative stem cells outside the end buds. The results show, first, that there is a continuity of markers from the epidermal epithelium from which the gland derives to the fully developed gland. The order of appearance of markers establishes a developmental pathway, which agrees with that previously established, although it is based on different criteria. The results also show that cells with markers of putative stem cells can be identified outside the end buds in sections of ducts and even in alveoli of lactating glands.

MATERIALS AND METHODS

Antibodies. The following mouse monoclonal antibodies were used: 2B4B2 (to M, 54,000 and 56,000 cytokeratins of luminal cells) (2); 1A10 (to a cytokeratin of myoepithelial cells) (2); 57B29 (to a surface antigen of myoepithelial cells) (1); 9B16 (to microvilli) (4); and 31G10 (to Thy-1.1) (5). Rabbit antiserum to purified chicken gizzard myosin and to total muscle keratin were a gift from Dr. J. Singer; rabbit antiserum to purified collagen IV was a gift from Dr. L. Lioota.

Immunofluorescence. Immunofluorescence was determined on unfixed cryostat sections using the sandwich technique as already described (1), adding p-phenylenediamine to the mounting medium to avoid fading (6). Double immunofluorescence was used whenever possible with a mouse monoclonal and a rabbit antisem. Double immunofluorescence with two monoclonals could not be carried out by either direct labeling (the antibodies lost their specificity) or by differential recognition by a second antibody (because all our monoclonals are of the G1 isotype). We were successful in using a destaining-re staining method (7). Tissue sections were attached to slides using poly-L-lysine; they were stained first with a monoclonal, used in conjunction with a rabbit antiserum, and mounted in glycerol-phosphate buffered saline-p-phenylenediamine. The sections were microscopically examined and photographed, using reference points to locate the structures. The slides were then soaked in phosphate-buffered saline for 5 min and the coverslips were removed. After washing twice in the same buffer, the sections were exposed to a glycine buffer (75 mM; pH 2.3) six times, 1 h each time. After washing with buffer, the sections were then stained with the new monoclonal. The completeness of stain removal was monitored in separate sections and also by the absence in the restained section of the stain generated by the rabbit antiserum in the first phase.

RESULTS

Prenatal Development. Mammary ducts could be recognized in rat fetuses beginning at the 17th day. Earlier stages were not
studied. In 17-day fetuses we recognized ducts that were brilliantly stained by three monoclonal antibodies: 1A10, 24B42 (specific for different cytokeratins) (2); and 57B29 (specific for an uncharacterized surface molecule). This overlapping of the markers is based on the recognition of the same duct in successive sections stained by the various antibodies; all cells were uniformly stained by any of them (Fig. 1, A–C), although 1A10 stain was poorer in some cells. All cells were negative for p-myo. These early ducts are surrounded by a basement membrane intensely stained by antibodies to purified collagen IV (not shown). During this period of late pregnancy (from the 17th day forward) the basal layer of the epidermis was brilliantly stained by 1A10 and 57B29 but was negative for 24B42 or p-myo (Fig. 1, D and E); so at this stage all cells of mammary ducts have two markers of the basal layer of the epidermis and in addition the cytokeratins defined by 24B42, which in the adult glands are confined to luminal cells and putative stem cells. The appearance of these keratins marks therefore the differentiation from epidermal epithelium to mammary epithelium. In the subsequent days of gestation the markers retained the same distribution.

Postnatal Development. In some ducts of the newborn rat the differentiation between luminal and myoepithelial lineages begins to emerge. Although most cells are still stainable by 1A10 and 24B42 (Fig. 1, F and G), isolated p-myo positive cells make their appearance close to the basement membrane. Most of these cells are still 24B42 positive and retain 1A10 staining. This is the beginning of myoepithelial differentiation. Another change is the disappearance or a great reduction of the 1A10 staining in a proportion of cells close to the lumen of some ducts; this is the beginning of luminal differentiation. Both the loss of 1A10 staining cells and increase of p-myo staining cells are more pronounced in the parts of ducts furthest away from the epidermis (Fig. 2). At this stage strong 57B29 staining is confined to the outer layers of ducts, mostly in cells that are also 1A10 positive; a few of them are p-myo positive (Fig. 3). Cells closer to the lumen show weaker staining (Fig. 3, A and B). Monoclonal 9B16, which recognizes microvillin (4) stains very weakly the luminal surface of some cells (not shown). At 3 wk the gland has reached the organization previously described (2, 4).

Because 57B29 stains the earliest mammary ducts, we have examined in detail its postnatal distributions. We find, as already reported, that p-myo positive cells in the ducts are strongly stained by 57B29. Strongly stained are also end bud cells whether or not they are p-myo positive (Fig. 3, C and D). These cells are also 24B42 positive. The luminal cells of ducts (Fig. 4, A and B) and ductules (Fig. 4, C and D) are also stained

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Fig. 1. A–C, 17-day rat embryo, mammary ducts. A, 57B29; B, 1A10; C, 24B42; D and E, rat epidermis with hair buds; D, newborn 57B29; E, 20-day embryo 1A10; F and G, newborn rat mammary ducts; F, 1A10; G, 24B42. × 150.
MARKER EVOLUTION DURING DEVELOPMENT OF RAT MAMMARY GLAND

Fig. 2. Mammary ducts of a newborn rat at various levels in double immunofluorescence. A and B, close to epidermis; C and D, middle; E and F, far from epidermis. A, C, and E, 1A10; B, D, and F, p-myo. × 150.

by 57B29 but more weakly. In lactating glands staining is confined exclusively to myoepithelial cells, especially their outer surfaces.

We have previously reported a possible association of p-myo and 24B42 positivity in the myoepithelial cells of some ducts. We can now confirm this association, eliminating the reservation of juxtaposition with luminal cells which was previously advanced (Fig. 4, E and F). These cells are also positive for 1A10. They are present mostly in glands of 3-wk-old animals, especially in ducts close to end buds, which are those formed most recently.

The association of 24B42 and 1A10 reactivity in cells of the outer layer of young ducts and end buds was previously established in an indirect way. Now we have confirmed it by the destaining and restaining method described under “Materials and Methods.” Cells positive for all three antibodies (24B42, 1A10, and p-myo) could be directly identified in end buds (Fig. 5, A–C) and in even greater abundance in young ducts (Fig. 5, D–F) at both 3 and 7 wk of age. Similar cells are also present in some parts of lactating glands.

Because reactivity with 1A10, 24B42, and 57B29 is a characteristic of the primitive mammary cells and because the study of lineages suggests that cells with these markers present in end buds are stem cells, we propose that the cells with the same markers present in the outer layer of young ducts should also be defined as stem cells for mammary development, although they have a different morphology.

We have been concerned with a possible developmental relatedness between myoepithelial and luminal cells. Two findings raise the possibility that myoepithelial cells contribute to the
formation of the luminal layer. One is the presence of thin appendages of luminal cells containing the keratin recognized by 24B42 squeezed between myoepithelial cells and reaching as far as the basement membrane (Figs. 6 and 7, A and B). The other is the staining of some luminal cells by a rabbit antiserum to total keratin, which stains strongly the myoepithelial cells (Fig. 7C). These occasional cells are always pear shaped, with the pointed end directed at the myoepithelial layer. These cells are also selectively stained by monoclonal 48B45 which was subsequently lost. They may correspond to the cells with appendages recognized by 24B42. We have never seen comparable cells stained by 1A10. We will call them intermediate cells.

We have studied all our material for possible expression of Thy-1 antigen. This approach was motivated by the finding that epithelial cells derived from rat mammary tumors generate in vitro Thy-1 positive cells (8, 9). The most consistent Thy-1
expression was found in epithelial cells of mammary ducts during the first postnatal week in the form of sparse grains at the cell surface (Fig. 8). We only exceptionally found Thy-1 expressing cells in 3- or 7-wk-old animals and only in the outer layer of end buds. Mature ducts have been consistently negative, both in the luminal and myoepithelial cells.

DISCUSSION

We have identified a set of markers that are suitable for characterizing different kinds of cell types in the rat mammary gland and have used them in the past for defining epithelial cell lineages and putative stem cells in the adult animal (1, 2). In the present work we have expanded this approach to include prenatal and early postnatal stages. Two main points emerge from this work, the nature and localization of putative stem cells for mammary development can be determined more precisely, and a new interpretation can be given for the role of myoepithelial cells in mammary development.

The results show that the earliest mammary ducts examined (at 17 days of gestation) have two markers characteristic of basal epidermal cells at the same stage of development, a surface antigen defined by monoclonal antibody 57B29 and a cytokeratin defined by monoclonal 1A10. In addition they have an antigen absent in the epidermal cells, the cytokeratins defined by monoclonal 24B42. All cells are stained by all three antibodies showing that at this stage the differentiation between luminal and myoepithelial cells has not yet taken place. Immunocytochemically demonstrable myosin, which is characteristic of
myoepithelial cells, is absent. Therefore only the 24B42 cyto-
k eratin is characteristic of mammary cells at this stage.

A differentiation between luminal and myoepithelial cells
appears at around the time of birth. In newborn animals, cells
displaying the myoepithelial cytokeratin defined by 1A10 tend
to be at the periphery of ducts, especially in their more distal
parts, and some of them are myosin positive; many luminal
cells have lost 1A10 and start displaying microvillin (4), de-
tected by monoclonal 9B16. 57B29 stains most strongly the
outer layer of the ducts but is still recognizable, although it is
much weaker, in luminal cells. 24B42 continues to stain all
cells. At this stage therefore the luminal and myoepithelial
lineages become defined: cells of the luminal lineage are
strongly stained by 24B42 and also display 9B16; cells of the
myoepithelial lineage are strongly stained by 1A10, 24B42, p-
myo, and 57B29. These differences become more accentuated
at 1 wk of age, when cells of the myoepithelial lineage are more
uniformly myosin positive and less regularly 24B42 positive.
The luminal lineage remains unchanged. At 3 wk the adult
pattern is established; ducts are terminated by end buds and
both contain various cell types as already described (1, 2). At
this stage the 57B29 monoclonal stains strongly end bud cells
and the myosin positive cells present in ducts but weakly or
moderately the luminal cells of ducts. In 7-wk glands luminal
cells lack this marker in the ductules, which are probably the
precursors of alveoli. The luminal cells of alveoli present in
lactating glands are not well stained by any of the antibodies.
This finding is in part explained by the way our monoclonal
antibodies were prepared, using as antigen cells derived from a
mammary carcinoma, the cells of which are probably related to
cells of early stages of the developmental pathway.

At 3 wk the young ducts have myoepithelial cells of elongated
morphology, which continue to be strongly stainable by 24B42,
1A10, 57B29, and p-myo. At 7 wk such cells are still present,
but are rare; most myoepithelial cells then are 24B42 negative.
The markers we have used make it possible to identify several
different cell types in the rat mammary gland and to suggest
how they are related to each other. The precise significance of

Fig. 6. Seven-wk-old rat in double immuno-
fluorescence. A and B, section through the
wall of a duct stained with 24B42 (A) and p-
myo (B). Note thin appendix of a luminal cell
reaching the outer surface of the myoepithelial
layer (arrows). C and D, tangential section
showing appendages of luminal cells through
interstices in the myoepithelial cell layer (ar-
rows). C is stained by 24B42, D by myosin. X
300.
Fig. 7. A and B, higher magnification of C, D, respectively, in Fig. 6. × 1250. C, longitudinal section through a duct stained with total muzzle keratin. Note stained pear-shaped cells in luminal layer. × 300. Arrows show position of thin appendage of luminal cell.

Fig. 8. One-wk-old rat in double immunofluorescence; section through a duct stained with 3IG 10 (A) or antibodies to purified collagen IV (B), × 500.

these markers in molecular terms is not known; we do not know whether in a cell negative for a certain marker the molecule is not made or its presence is masked. We have shown masking owing to filament disaggregation of the 24B42 keratin markers in cultured mammary cells (10). The great uniformity of the markers in cells of similar location and developmental stage in the present work shows that masking, if it occurs, is itself a developmental characteristic.

We have previously suggested that the myoepithelial and luminal lineages converge in some end bud cells which are positive for 24B42 (or Le61 which has similar distribution) and p-myo. We have now shown that these cells are also strongly positive for 1A10 and 57B29. These results strengthen the previous suggestion because these cells have all the markers of the primordial mammary cells, with the addition of myosin. We have also established that cells with these markers are not confined to the end buds. They constitute most of the myoepithelial layer of young ducts, close to the end buds, and they are scattered in the myoepithelial layer of older ducts and also in some alveoli of lactating glands.

The suggestion that these cells, or a closely related type perhaps not stained by p-myo are stem cells of mammary development is reinforced by the results of transplantation in mice, which showed that stem cells capable of giving rise to a complete gland are present both in segments of ducts disconnected from end buds and in cells derived from late pregnant or lactating rats, which lack end buds (see Footnote 4).

Previously, attempts at defining mammary stem cells and their developmental potential were made in vitro using cultures of cell derived from mammary carcinomas that give rise to a variety of cell types (11, 12). The marker distribution of these cells does not correspond to any of the cell types we can identify in the normal mammary gland, perhaps because growth in culture profoundly affects the expression or detection of markers, as clearly shown for cytokeratins (10). The significance of the developmental potential of these cultivated cells is obscured by the fact that they are cancer cells; the changes might well be an expression of cancer progression rather than of development. The cells arising in vitro have no clear correspondence to normal cell types. For instance, the putative myoepithelial cells arising in the cultures have none of the markers of myoepithelial cells observed in vitro and have abundant Thy-1 (8) which myoepithelial cells do not have.

The results we obtained raise the question of what the role is of the cells of the myoepithelial layer. At the tips of end buds these cells show little differentiation based not only on marker distribution but also on ultrastructure (13). In the lower parts of the end buds and in the ducts, they progressively acquire the characteristic longitudinally elongated shape and marker distribution. Around the ducts they form a closely packed layer. Around the ductules they have irregular shape, and around the lactating alveoli they have the characteristic basket shape. That these cells may not simply have a contractile function is suggested by their multiplicative activity, which is the highest of all cells in ducts, much higher than that of luminal cells and by their active multiplication where small branches (ductules) are formed on ducts (15). The present evidence suggesting that in end buds and ducts some cells of this layer perform the function of stem cells is in keeping with their intense multiplication in the growing gland.

It is probable that the myoepithelial layer contains not only the stem cells for mammary development but also another kind of pluripotent cell, whose existence is suggested by graft experiments in mice (see Footnote 4), capable of generating ductules...
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Cell Type

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and in pregnancy alveoli. These cells, however, are not characterized. The myoepithelial layers may therefore constitute an evolving system, as also suggested by the changing morphology of the cells, containing mammary stem cells, precursors of alveoli, and mature myoepithelial cells, with different distribution at different levels of the ductal tree.

The mature myoepithelial cells, which exist only around the alveoli of lactating glands, differ from other cells of the same lineage not only for their profound morphological difference but also for the absence of 24B42 reactivity and near disappearance of 57B29 reactivity.

Myoepithelial cells probably also contribute to forming the luminal layer. This possibility is supported by the presence in the ducts of intermediate cells with the body in the luminal layer and appendages reaching the basement membrane; these cells display distinctive markers, one of which is present also in the basal layer of ducts and in the immature cells of the end buds. Luminal cells are not, however, terminally differentiated cells because they multiply (15).

We suggest it would be useful to adopt a designation that distinguishes the myoepithelial cells present in end buds and ducts from those present in alveoli. The former could be called “basal” cells, as used in describing mammary tumors (14), leaving the term “myoepithelial” for those of alveoli. The term basal also emphasizes the close relatedness of these cells to the cells of the basal layer of the epidermis from which the mammary gland derives.

We summarize the present findings and those previously presented in the scheme of Fig. 9.

REFERENCES

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