Epithelial Characteristics of Five Subpopulations of a Heterogeneous Strain BALB/cfC3H Mouse Mammary Tumor

Jean C. Hager, Jose Russo, Roberto L. Ceriani, Jerry A. Peterson, Suzanne Fligiel, Grant Jolly, and Gloria H. Heppner

Abstract

We have described previously the isolation and characterization of five distinct subpopulations of tumor cells from a single spontaneous strain BALB/cfC3H mouse mammary tumor (Cancer Res., 38: 3174-3181, 3758-3763, 1978). Subpopulations 68H and 4.10 are polygonal and grow in epithelioid patterns in vitro, whereas subpopulations 66, 67, and 168 are fusiform and grow in lattice or fibroblast-like patterns. Line 4.10 produces tumors with distinctly glandular architecture, whereas the other four subpopulations produce poorly differentiated tumors with mixed epithelial-sarcomatous histological patterns. All five lines were evaluated for epithelial characteristics. Dome formation, characteristic of transporting epithelial cells, could be induced by dexamethasone or dimethyl sulfoxide only in line 4.10 cells. Antibodies to cell type-specific mammary epithelial antigens reacted with each of the subpopulations. All five subpopulations had ultrastructural features of epithelial cells, including desmosomes (all five lines), junctional complexes (68H, 4.10, early-passage 66 and 67 only; poorly defined in 168), and growth in cords demonstrating polarity (68H cells). Less definitive myoepithelial characteristics were also seen in four of the lines, including an incomplete reaction for Na+-K+-ATPase (4.10 cells), hemidesmosome-like junctions (168 and early-passage 66 cells), and pinocytotic vesicles at lower than normal frequency (66, 67, and 168 cells). Thus, none of the lines were distinctly myoepithelial. We conclude that the five subpopulations are epithelial cells that express a spectrum of epithelial characteristics.

Introduction

Evidence that mammary and other solid tumors of animals and humans are heterogeneous has come from many laboratories (reviewed in Refs. 17 and 19). A mouse mammary tumor model has been developed in our laboratory to study the origin and consequences of intratumor heterogeneity (5, 10, 14, 15, 17-19, 32). Four subpopulations of tumor cells (lines 66, 67, 168, and 68H) were isolated from a single primary strain BALB/cfC3H mouse mammary tumor that was heterogeneous in regard to histology, cellular morphology, karyotype, and mouse mammary tumor virus antigen expression (10). A fifth subpopulation (line 4.10) was isolated from a metastatic tumor nodule in the lung of a mouse bearing the tenth in vivo passage of the parent tumor (18). These subpopulations differ among themselves in karyotype, in vitro growth, and morphological characteristics (10, 17-19). They are all tumorigenic in syngeneic mice but differ in the tumorigenic cell inoculum required to produce tumors in 50% of the animals, latency period, growth rate, immunogenicity, ability to metastasize, and sensitivity to chemotherapeutic agents (10, 14, 17, 18, 25).

In addition to supporting tissue and fat, the mammary gland is composed of epithelial and myoepithelial cells, both of which have been reported to give rise to tumors (3, 16, 44). It is not known if the heterogeneity observed in tumor cell populations is a consequence of neoplastic transformation in more than one of the cell types comprising the mammary gland or, rather, is a consequence of the expression of the biological potential of pluripotent neoplastic cells (4, 15, 30, 31, 34, 46), irrespective of whether the tumor had a mono- or multicellular origin (13, 33).

The purposes of the work described in this paper were to investigate the epithelial nature of the 5 tumor subpopulations isolated from a single mammary tumor in order to shed light on the basis for intratumor heterogeneity. The epithelial characteristics evaluated were dome formation (1, 22-24, 32), expression of mammary epithelial antigens (7-9, 45), and presence of ultrastructural characteristics such as desmosomes, junctional complexes (11, 12, 32), and cell polarity (38). These characteristics, taken all together, are considered as evidence for epithelial, rather than stromal, origin of cells from mammary glands and tumors (32).

Materials and Methods

Mice. Our BALB/cfC3H mice are the progeny of a breeding pair obtained in 1969 from the Cancer Research Laboratory, University of California, Berkeley, Calif., and since maintained by brother-sister mating. The BALB/cfC3H mice carry mouse mammary tumor virus.

Origin and Maintenance of Tumor Subpopulations. The derivation of lines 66, 67, 168, 68H, and 4.10 has been described previously (10, 18). Lines 66, 67, 168, and 68H were derived from the first in vitro passage of a single mammary tumor that arose spontaneously in a strain BALB/cfC3H breeding female (10). Line 4.10 was derived from a single spherical nodule, approximately 1 mm in diameter, that was isolated from the right medial lobe of the lung of a BALB/cfC3H mouse bearing an s.c. implant of cells of the tenth in vivo passage of the parent tumor from which the other subpopulations were derived (18).
Cells are routinely plated in Falcon tissue culture dishes (Falcon Plastics, Oxnard, Calif.) in Waymouth's medium supplemented with 15% heat-inactivated fetal calf serum, L-glutamine (2 ml), gentamycin (20 µg/ml; Schering Corp., Port Readings, N. J.), penicillin (100 units/ml), and streptomycin (100 µg/ml) and buffered with NaHCO3. Unless otherwise noted, all cell culture reagents are purchased from Grand Island Biological Co., Grand Island, N. Y. When subcultures have grown to a monolayer, the cells are harvested with 0.125% trypsin and 0.5% EDTA in 0.9% NaCl solution and centrifuged for 5 min at 450 × g. The pellet is resuspended in fresh medium, and the cells are plated in Falcon culture dishes and grown at 37°C in a 5% CO2 in air atmosphere.

Dome Formation. Four different culture conditions were used to induce dome formation: (a) high-density culture of cells on plastic surfaces (1, 29); (b) culture medium supplemented with 10^-6 M dexamethasone (24); (c) culture medium supplemented with 10 µg insulin per ml and 10 µg hydrocortisone per ml (22-24); and (d) culture medium supplemented with 1% dimethyl sulfoxide.3 Inducers were added 24 hr after plating, and the antibody was precipitated by 40% ammonium sulfate, later, 3 mg more of the fat-free globules were injected. Ten days later, 3 mg more of the fat-free globules were injected. Ten days later, the rabbits were bled, the serum was separated, and the antibody was precipitated by 40% ammonium sulfate, pH 7.0. The precipitated pellet was redisolved to its original volume, dialyzed against PBS, heated for 1 hr at 56°C, and sequentially absorbed 1:1 with the following murine cell types: RBC; fetal fibroblasts; liver cells; and kidney cells. After absorption, the antibody was purified by affinity chromatography. The affinity reagent was prepared by homogenizing lactating BALB/c mouse mammary gland, adding glutaraldehyde to a final concentration of 0.2%, allowing fixation while stirring for 60 min, and collecting particulate material by centrifugation. The particulate material was washed with PBS, resuspended, allowed to stand for 60 min in 0.5% glycine, and washed again in PBS. Five ml of the glutaraldehyde-fixed tissue were placed in a column, and 1 ml of absorbed antibody was poured into it and allowed to bind for 30 min. The column was washed in PBS, and the bound anti-mouse mammary epithelial antibody was eluted with 4 ml of 2 M NaSCN. The eluant was dialyzed, and the purified antibody was brought to its original volume (1.0 g/100 ml) for protein determination and titration by dilution and immunofluorescence.

Identification of Cells by Indirect Immunofluorescence. Cells were removed from cultures by trypsinization, and the cells were allowed to recover for 48 hr by plating on an agar substrate in culture dishes (8). The cells were then collected and washed at room temperature 3 times with a Ca2+-Mg2+-free 0.9% NaCl solution containing 0.5% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) and 0.01 M sodium azide. Twenty µl of anti-mammary epithelial antibody were added to a pellet of 1 × 10^6 cells, mixed well, and incubated for 30 min in a 37°C water bath with intermittent agitation. The cells were washed 3 times and pelleted, and 20 µl of fluorescein-labeled goat anti-rabbit antibody (Antibodies Inc., Davis, Calif.) diluted 1:10 were added. The cells were mixed and incubated again for 30 min at 37°C. The cells were washed again 3 times, pelleted, and placed on a glass slide, mounted under a coverslip. Slides were viewed with a Lietz Dialux microscope equipped with fluorescence epillumination (100-watt tungsten halogen lamp) with one KP 500 excitation filter and one K350 barrier filter. The intensity of the reaction was scored on a scale of + to +++++ in which the latter is the greatest and is the type of reaction observed with normal mouse mammary gland epithelial cells (7, 8). The percentage of antigen-expressing cells was determined by counting 500 cells.

Scanning Electron Microscopy. Cells were grown in Falcon culture dishes on prescored glass coverslips and fixed when subconfluent according to the procedure of Hirsch and Fedorko (20) in 1.25% glutaraldehyde in 0.1 M cacodylate, 1% aqueous OsO4 in 0.1 M cacodylate for 60 min on ice. The cells were washed in 0.1 M cacodylate and then in 0.85% NaCl solution and were dehydrated in a graded (30 to 100%) ethanol series. The cells were dried by the critical-point method, coated with a gold palladium alloy (60:40), and observed in an ETEC Autoscan electron microscope at an accelerating voltage of 20 kV.

Transmission Electron Microscopy. Cells were grown in Falcon culture dishes and processed when subconfluent. Early-passage cells were scraped from the culture vessel with a rubber policeman, collected as a 1-mm-thick pellet by centrifugation, and fixed in 1.25% glutaraldehyde in 0.1 M sodium cacodylate for 2 hr at 4°C. The fixed pellet was cut into 1-cm^2 pieces and postfixed in 1% OsO4 in 0.1 M sodium cacodylate, dehydrated in a graded ethanol series and propylene oxide, and infiltrated and embedded in Epon 812 (60:40), and observed in an RCA EMU4 electron microscope at 75 kV. Later-passage cells were fixed in situ in the culture dishes by the fixation method of Hirsch and Fedorko (20) in a fixative consisting of 1.0 M disodium ATP from equine muscle (Sigma), 100 mM NaCl, 15 mM KCl, 3.6 mM Pb(NO3)2, and 88 mM Tris-maleate buffer (pH 7.2) containing 1% sucrose, dehydrated in a graded ethanol series and propylene oxide, lifted off the plastic culture dish, infiltrated with propylene oxide, transferred and embedded in an epon-araldite mixture (27), and polymerized at 60°C for 48 hr. Thin sections were cut with diamond knives, stained, and examined with a Siemens 1A electron microscope at 60 kV.

Histochemical Localization of Na+-K+-ATPase. Cultured cells were fixed when subconfluent as described for transmission electron microscopy and incubated for 20 min at 37°C in a mixture consisting of 1.0 M disodium ATP from equine muscle (Sigma), 100 mM NaCl, 15 mM KCl, 3.6 mM Pb(NO3)2, and 88 mM Tris-maleate buffer (pH 7.2) containing 6% sucrose (39, 40). Cells were postfixed in 1% OsO4 in 0.1 M cacodylate for 3 hr at 4°C, dehydrated, and embedded in epon-araldite as described above. One-µm-thick sections were cut with glass knives in an LKB Pyramitome and stained with 0.5% toluidine blue. Slides were viewed with a Lietz Dialux microscope equipped with fluorescence epillumination (100-watt tungsten halogen lamp) with one KP 500 excitation filter and one K350 barrier filter. The intensity of the reaction was scored on a scale of + to +++++ in which the latter is the greatest and is the type of reaction observed with normal mouse mammary gland epithelial cells (7, 8). The percentage of antigen-expressing cells was determined by counting 500 cells.

3 H. Soule, unpublished results.
4 The abbreviations used are: DMBA, 7,12-dimethylbenz(a)anthracene; PBS, phosphate-buffered saline (0.85% NaCl, 0.01 M phosphate, pH 7.2).
blue. The sections were examined in phase contrast for the detection of positive reaction sites (39–41). Areas with positive reactions were then sectioned for electron microscopy, stained with uranyl acetate and lead citrate (35), and examined with a Siemens 1A electron microscope at 60 kV.

RESULTS

Dome Formation. The ability of the 5 tumor subpopulations to form domes in high-density cell culture on an impermeable substrate was investigated both in conventional culture medium and after treatment with several inducers. None of the lines spontaneously formed domes in high-density cultures. Only 4.10 could be induced to produce a few domes/culture after 48 hr of treatment with dexamethasone or dimethyl sulfoxide.

Expression of Mouse Mammary Epithelial Cell Antigens. All of the tumor subpopulations reacted positively in immunofluorescence assays with antibodies specific for mouse mammary epithelial cell surface antigens. From 80 to 90% of the cells were positive in 4 of the subpopulations; however, in line 67, only 65% of the cells were positive. Line 66H and 4.10 cells were the most intensively reactive (+ + +), and line 66 and 168 cells were intermediate in reactivity (+ +), and line 67 cells were the most intensively reactive (+ + +) but less so than normal mammary gland epithelial cells (+ + + +). Line 67 cells were intermediate in reactivity (+ +), and line 66 and 168 cells were less reactive (+).

Ultrastructure. Each of the tumor subpopulations was examined by transmission electron microscopy at early passage (levels 3 to 12) and by both transmission and scanning electron microscopy at late passages (after 3 years in culture). The characteristics are summarized in Table 1.

Line 66. Line 66 cells appear highly disperse in monolayer and grow with a lattice pattern, although they achieve confluence and pile up in multiple layers. Line 66 cells are elongated in shape, and their nuclei are oval with slight indentations. In early-passage cells, a single nucleolus, and moderate amounts of heterochromatin are observed. The cytoplasm is mildly electron dense, containing scarce amounts of smooth and rough endoplasmic reticulum, mitochondria, and abundant ribosomes. No virions have been observed. At the seventh passage level, the cell surface was sparsely covered with microvilli and had a few pinocytotic vesicles. Hemidesmosome-like junctions (Fig. 2) were the most frequently seen junctions in early-passage cells, although desmosomes and junctional complexes were also observed. In contrast, at later passages, neither junctional complexes nor hemidesmosomes are present, and the cells have a smooth surface (Fig. 3). The nuclei of later-passaged cells are more elongated than those of earlier passages and contain more fine chromatin (Fig. 4). Bundles of microfilaments similar to tonofilaments are usually observed in the cytoplasm running parallel to the long axis of the cells.

Line 67. Line 67 cells are fusiform and grow in a lattice pattern, similar to that of line 66 cells, with little cell-to-cell contact. The cytoplasm contains abundant ribosomes, few round mitochondria, and scantly rough and smooth endoplasmic reticulum (Fig. 5). Lysosomes are frequently found containing viral particles (Fig. 6). The surfaces of early-passage line 67 cells were covered with blunt microvilli with budding type B retrovirus particles, and adjacent cells were joined by desmosomes and junctional complexes (Fig. 7). In contrast, in late-passage cells, the plasma membranes show no virions and little activity, other than a small number of pinocytotic vesicles.

Table 1

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>Desmosomes</th>
<th>Junctional complexes</th>
<th>Virus production</th>
<th>Hemidesmosomes</th>
<th>Pinocytotic vesicles (cav., s)</th>
<th>Myofilaments</th>
<th>Na⁺-K⁺-ATPase</th>
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<tr>
<td>Line 66</td>
<td>+</td>
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<td>Line 67</td>
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<td>Line 168</td>
<td>+</td>
<td>ill-defined</td>
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<td>+</td>
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<td>—</td>
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<tr>
<td>Line 68H</td>
<td>+</td>
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<tr>
<td>Line 4.10</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Normal mammary gland epithelium</td>
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<tr>
<td>Normal mammary gland myoepithelium</td>
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<td>+</td>
<td>+</td>
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<td>Normal stromal fibroblasts</td>
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* Lost or diminished in late-passage cells.

* Distinguishable from observation in normal counterpart cells (see text for details).

Line 68H. Line 68H cells are predominantly polygonal in shape and grow in typical epithelioid cords. Nuclei are round...
to oval with an irregular outline, dispersed heterochromatin, and one or 2 nucleoli. The cytoplasm is of intermediate electron density with moderate amounts of mitochondria, abundant ribosomes and microfilaments, and scanty smooth and rough endoplasmic reticulum. There are abundant microvilli on the free surface of the cell, as well as between adjacent cells (Fig. 14). Type B retrovirus particles are seen budding from the cell surface and from the tips of the microvilli, as well as free in the intercellular spaces (Fig. 15). Intracytoplasmic virus particles have not been seen. Adjacent cells are joined by well-developed desmosomes and junctional complexes (Fig. 14). Scanning electron microscopy at later passage demonstrates an epithelial growth pattern (Fig. 16). The cells are slightly more spindle shaped than the early-passage cells although the cytoplasmic features are similar (Fig. 17).

**Line 4.10.** Line 4.10 grows in a typical epithelioid paving stone pattern forming a single monolayer. Individual cells are roughly cuboidal and display varying numbers of short blunt microvilli (Figs. 18 and 20). The nuclei are ovoid to round with few infoldings in the nuclear membrane and have a small amount of heterochromatin. Frequently, one nucleolus/ nucleus is seen. The cytoplasmic features are the same ones described for 68H and 168 cell lines. Adjacent cells are joined by desmosomes, and junctional complexes are detected near the free surface of the cells (Fig. 19). Extracellular type B virions but not budding forms were observed at 12th passage (Fig. 21). At later passages, only little budding virus activity was observed.

**Histochemical Localization of Na⁺⁻K⁺-ATPase Activity.** Using a histochemical technique (39-41), the 5 tumor subpopulations were examined for the presence of Na⁺⁻K⁺-ATPase, a characteristic marker for myoepithelial cells in the resting mammary gland. Lines 66 and 67 are negative. Lines 168 and 68H are negative to weakly positive. Cells of line 4.10 are strongly positive at the terminal or free surface (Fig. 22) but negative at the basal border.

**DISCUSSION**

Previous publications from our laboratory have described the isolation of distinct subpopulations of tumor cells from a single mammary neoplasm (10, 14, 15, 17-19, 25). Although cells of 2 of the subpopulations (lines 4.10 and 68H) are polygonal and grow in typical epithelioid patterns in vitro, cells of the other 3 subpopulations (lines 66, 67, and 168) are fusiform in shape and grow in lattice-like networks (lines 66 and 67) or a sheetlike fibroblastic pattern (line 168). The original tumor from which lines 66, 67, 168, and 68H were isolated was a well-differentiated adenocarcinoma (10). The tumors produced by the s.c. inoculation of 4 of the lines are, however, poorly differentiated neoplasms with mixed intermingled epithelial-sarcomaous patterns (10). In contrast, line 4.10 produces tumor with prominent glandular patterns (18). Thus, the nature of line 4.10, and probably line 68H, is epithelial, whereas the nature of the other 3 subpopulations is uncertain. Lines 66, 67, and 168 might be derived from stromal fibroblasts, myoepithelial cells, or epithelial cells that assume fusiform morphology in vitro and partially differentiate in vivo. To distinguish among these possibilities, we utilized a spectrum of markers for mammary epithelial cells and myoepithelial cells (1, 22-24, 26, 32, 39-41).

Dome formation is considered to be an epithelial cell characteristic in vitro (1, 2, 21, 23-24, 32). Domes are transient blisters that form as a result of fluid transport across a confluent cell sheet. Dome formation depends on the presence of an intact permeability barrier that can be provided by epithelial tight junctions (6, 11, 26, 29, 32). None of the subpopulations form domes under high-density culture conditions, and only 4.10 forms domes under any conditions reported to induce dome formation in various types of transporting epithelial cells (22-24, 29).

Mouse and human mammary epithelial cells have been shown to express cell type-specific membrane antigens that can be recognized by antibodies prepared against the milk fat globules of the appropriate species (7, 9, 45). These mammary epithelial antigens are not present on either mammary gland fibroblasts (7) or myoepithelial cells (7) or on other murine tissues (7, 8). Mammary epithelial antigens are present on mammary epithelial cells in culture and after neoplastic transformation (8). The antibodies to mouse mammary epithelial cell antigens react with cells of each of the 5 subpopulations, indicating the presence of epithelial cell antigens.

Epithelial and myoepithelial cells can also be distinguished from each other and from fibroblasts by ultrastructural characteristics (28, 32). The plasma membranes of epithelial cells, in contrast to stromal fibroblasts, are specialized to form desmosomes and junctional complexes between cells. Line 68H, 4.10, and 67 cells possess both desmosomes and junctional complexes, line 168 cells have desmosomes but ill-defined junctional complexes, and line 66 cells have desmosomes but no evident junctional complexes. Other features of epithelial cells (32, 39) observed in line 68H are the growth of cells in cords and demonstration of polarity with luminal cell borders covered with microvilli from which mammary tumor virus buds. Therefore, lines 68H, 4.10, and 67 appear to be epithelial cells by ultrastructural criteria. Lines 66 and 168 have some characteristics of epithelial cells but lack others. All are clearly distinguished from fibroblasts by their surface specializations.

Myoepithelial cells are positive for Na⁺⁻K⁺-ATPase and are characterized by the presence of myofilaments, pinocytotic vesicles, and hemidesmosomes (28, 32, 42). Myofilaments have not been seen in any of the lines. Hemidesmosome-like formations are observed in early-passage line 66 cells and in cells of both early and late passages of line 168. They can be distinguished from hemidesmosomes observed in normal myoepithelial cells (41, 42) and are not associated with basement membrane. Pinocytotic vesicles are observed in cells of lines 66, 67, and 168, although less frequently than in normal myoepithelial cells (41). Although line 4.10 cells are distinctly positive for Na⁺⁻K⁺-ATPase at the surface free borders, a characteristic that may be related to their ability to form domes (1, 36), they are negative at the basal borders, in contrast to myoepithelial cells in which all of the cell membrane is positive (41, 42). Appearance of Na⁺⁻K⁺-ATPase activity has been reported to accompany neoplastic transformation in some mouse mammary epithelial cells (43). None of the other subpopulations are positive in this test. Thus, although some myoepithelial characteristics can be seen in 4 of the 5 subpopulations, they are not prominent, and their presence does not clearly define any subpopulation as being myoepithelial. In this

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5 R. L. Ceriani, unpublished results.
regard, the subpopulations are reminiscent of the "indeterminant" cells in human breast cancer discussed by Ozello (28). Line 16B, 68H, and 4.1 cells also have ultrastructural features similar to the intermediate cell of the rat mammary gland (37), which is the cell that responds to DMBA administration by proliferating to form the main subpopulation of the DMBA-induced tumor (39). The subpopulations are also similar ultrastructurally to Rama 25, a rat mammary epithelial tumor "stem" cell line which has been isolated from a DMBA-induced mammary tumor (1, 36). Rama 25 can differentiate into both fusiform cells which are myoepithelial and tumorigenic and those which are nontumorigenic variants (1, 36).

As a whole, the observations reported here are evidence that the 5 subpopulations of tumor cells isolated from a single strain BALB/cfC3H mouse mammary tumor are all epithelial cells, representing a spectrum of epithelial differentiation. The lines range from poorly differentiated 66 and 16B to moderately differentiated 67 and well-differentiated 68H and 4.10. Furthermore, it is clear from this and other discussions (32) that, although markers to distinguish epithelial, myoepithelial, and stromal cells may be definitive in the normal mammary gland or even in some tumors (28, 32), these markers are less definitive in many tumors. Any of the epithelial characteristics may be missing in epithelial tumor cells (e.g., dome formation). This would suggest that the basis for the differences among the subpopulations isolated from a single tumor lies in the expression of the potential of epithelial cells rather than transformation of myoepithelial or stromal components of the gland.

As we have described elsewhere (15), line 68H may provide a model to understand the origin of tumor cell subpopulations in that it is capable of generating a spectrum of distinct tumorigenic variants under physiological in vivo conditions. Some of these variants are fusiform and some polygonal in vitro, and certain cloned polygonal variants are also capable of generating further variants (15). Line 68H cells are distinct from the poorly differentiated proliferating "stem cells" described by Pierce et al. (31) and Wylie et al. (46) in mouse mammary tumors, since 68H cells are ultrastructurally well differentiated and are poorly proliferative (10). Line 68H cells more resemble Rama 25 cells (1, 36) and the highly differentiated cells of the rat pancreatic acinar carcinoma (34) that have been shown recently to give rise to less well-differentiated tumor cell types.

We suggest that tumor heterogeneity may arise by the production of variants by such progenitor cells, a process which may be continuous during tumor development and which provides a basis for selection and progression of neoplastic characteristics. This concept of tumor heterogeneity neither requires nor excludes a multicellular origin for neoplasia.

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REFERENCES


Fig. 1. Seventh-passage line 66 cells, × 9,000.

Fig. 2. Hemidesmosome-like junctions (arrows) in early-passage line 66 cells. × 10,400.

Fig. 3. Scanning electron micrograph of line 66 cells in late passage. × 1,000.

Fig. 4. Line 66 cells in late passage; compare with Fig. 1. × 9,000.
Fig. 5. Fourth-passage line 67 cells. × 6,000.
Fig. 6. Type B viral particles in the extracellular space and inside of lysosomes of line 67 cells. × 10,400.
Fig. 7. Cell surface of line 67 in fourth passage showing microvilli and desmosomes. × 30,000.
Fig. 8. Scanning electron micrograph of line 67 cells in late passage. × 1,000.
Fig. 9. Line 67 cells in late passage; compare with Fig. 5. × 6,000.
Fig. 10. Third passage of line 168 cells. x 9,000.
Fig. 11. Scanning electron micrograph of line 168 cells in late passage. x 1,000.
Fig. 12. Pinocytic vesicles in the free cell border and type B virions in the extracellular space of line 168 cells. x 30,000.
Fig. 13. Line 168 cells in late passage x 9,000.
Fig. 14. Typical epithelial cells of line 68H in early passage. × 6,500.
Fig. 15. Type B retrovirus particles budding from the cell surface of line 68H cells. × 30,000.
Fig. 16. Scanning electron micrograph of line 68H cells in late passage. × 1,000.
Fig. 17. Line 68H cells in late passage. × 6,500.
Fig. 18. Early passage of line 4.10 cells. × 9,000.
Fig. 19. Desmosomes near the free surface of line 4.10 cells. × 18,000.
Fig. 20. Scanning electron micrograph of line 4.10 cells in late passage, growing in an epithelioid paving stone pattern forming a single monolayer. × 1,000.
Fig. 21. Extracellular and budding type B virus from line 4.10 cells. × 30,000.
Fig. 22. Strong positive reaction for Na⁺-K⁺-ATPase activity at the free cell surface of line 4.10 cells. × 30,000.
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