In Vitro Differentiation and Progression of Mouse Mammary Tumor Cells

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ABSTRACT

We have isolated clonal cell lines from a transplanted adenocarcinoma induced by the RIII strain of mouse mammary tumor virus in a BALB/c mouse. Three major morphological cell types of these lines are developmentally linked; polygonal cells give rise to cuboidal and then to elongated cells.

All cell lines expressed markers that are characteristic of mammary basal cells. In addition, the polygonal lines contained cells that have cell markers and ultrastructural features of epithelial cells; in these lines an occasional cell was found with myoepithelial features. The cuboidal and elongated lines lacked many epithelial differentiation characteristics and showed no myoepithelial differentiation.

The cell lines contained variable numbers of acquired mouse mammary tumor virus and ecotropic murine leukemia virus proviruses. The various subclones derived from the original cell lines contained, in addition to the acquired proviruses of the parental line, one or more unique proviruses of either mouse mammary tumor virus or ecotropic murine leukemia virus origin. These unique insertions were used as genotypic markers to demonstrate the clonal relationship of the cell lines.

Both polygonal and elongated cells are tumorigenic and give rise to adenocarcinomas and sarcoma-like tumors, respectively. In contrast, the cuboidal cells are poorly tumorigenic. Since cuboidal cells are derived from the polygonal cells, this suggests that tumor progression in this system proceeds via intermediates that are either poorly or nontumorigenic.

INTRODUCTION

Mammary tumors induced by the MMTV3 in laboratory mice are of glandular origin (1). These tumors, when serially transplanted in syngeneic animals, can develop into highly malignant sarcoma-like tumors. Primary tumors containing areas of sarcoma-like growth are also found. Furthermore, sarcomatous changes have been observed in rat mammary tumors. Thus, the transformation of adenocarcinomas into sarcoma-like tumors seems to be a rather common feature of murine mammary tumors. Nevertheless, not all adenocarcinomas exhibit this change in phenotype: some retain their original morphology for many transplant generations (2). The sarcomas derived from epithelial cells are morphologically indistinguishable from fibrosarcomas originating at other sites, and they are composed of a single elongated cell type.

Related phenomena have been demonstrated in tissue culture. Primary cultures from mouse mammary tumors predominantly contain cells with a polygonal phenotype. These polygonal cells have retained many characteristics of epithelial cells, e.g., expression of keratin filaments, surface microvilli, desmosomes, and formation of domes. They are tumorigenic and give rise to adenocarcinomas. Cultures of polygonal cells can phenotypically change into elongated cells after prolonged periods of growth in vitro. These cultures, when injected into syngeneic mice, produce tumors composed of elongated cells resembling fibrosarcomas. Both from virally induced mouse mammary tumors (3–5) and from chemically induced rat mammary tumors (6–9), elongated cell lines have been isolated. They were obtained from cultures of cloned polygonal cells and thus are of epithelial rather than of mesenchymal origin. Nevertheless, the nature of the elongated cells remains uncertain. It has been argued that these cells actually represent a cell type with fibroblast-like properties which has not yet been recognized in the mammary gland (7, 9). Others have proposed that these cells represent a myoepithelial differentiation pathway (6, 9).

In our investigations on the development of the normal mouse mammary gland, we have generated a set of monoclonal antibodies against differentiation antigens. Based on the reaction pattern of these monoclonal antibodies, we could recognize five distinct cell types, one basal, one myoepithelial, and three different epithelial cell types (10).

We now describe the use of these monoclonal antibodies in the characterization of cell lines established from a mammary tumor. We isolated three distinct cell types (polygonal, cuboidal, and elongated) from a transplanted adenocarcinoma induced by the RIII strain of MMTV in a BALB/c mouse. We have investigated the developmental relationship between these three cell types and describe the properties of the individual cell types with respect to their differentiation state, epithelial characteristics, tumorigenic potential, and the histopathology of the tumors.

MATERIALS AND METHODS

Primary Cultures from Mouse Mammary Tumors. A BALB/c adenocarcinoma induced by the RIII strain of MMTV was excised and minced into small pieces with scalpel blades, resuspended in warm PBS, transferred to a 50-ml tube, and left for 1 to 2 min at room temperature to allow clumps to settle at the bottom. The supernatant, mainly containing red blood cells and cell debris, was aspirated, and the sediment was transferred to a culture flask. The pieces were then dispersed with 0.25% trypsin-0.02% EDTA in PBS for 5 to 10 min to yield a mixture of cell clumps and single cells. Trypsinization was stopped by the addition of 5% fetal calf serum, and large clumps were removed by passing the mixture through a gauze. The resulting cell suspension was centrifuged, resuspended in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, insulin (10 μg/ml), and hydrocortisone (50 ng/ml) (culture medium), and plated in Falcon tissue culture flasks at a cell density of 1 × 10⁶ cells/cm². For passage or subculture, cultures were incubated with trypsin until the cells had detached. The cell suspension was transferred into new dishes at a 1:4 split ratio.

Cloning of Cells by Limiting Dilution. Cultures derived from a transplanted adenocarcinoma were dispensed by trypsin into a single cell suspension that was then diluted to 50 or 5 cells/ml. Volumes of 100 μl were added to 96-well microtiter plates to give cell concentrations of 5 or 0.5 cells/well, respectively. The cells were first cloned at 5 and then twice at 0.5 cells/well. Elongated cells, obtained from the same tumor line, but of a different transplant generation, were used as feeder cells. To block their growth, these cells were exposed to mitomycin C for many transplant generations (2). The sarcomas derived from epithelial cells are morphologically indistinguishable from fibrosarcomas originating at other sites, and they are composed of a single elongated cell type.

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3 MMTV, mouse mammary tumor virus; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; MuLV, murine leukemia virus; LTR, long terminal repeat; SSC, standard saline citrate.

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Cloning Efficiency of Cells in Soft Agar. Cultures were dispersed with trypsin and resuspended in 0.25% agar (Difco) in culture medium at concentrations of 2.5 × 10^5, 2.5 × 10^6, and 2.5 × 10^7 cells/ml. Four ml of the cell suspension were layered on 3-ml bases of 0.5% agar in culture medium. Cloning efficiencies were determined by counting the colonies in duplicate 5-cm dishes 4 wk after initiation of the culture. Selected isolated colonies were removed with a micropipet, plated in 1.6-cm wells in Falcon culture dishes, and passed successively.

Saturation Densities and Doubling Times. For determination of doubling times, 1 to 2 × 10^6 cells were seeded per 5-cm dish, and cells were grown in 5 ml of culture medium. Two dishes were collected separately each day, and the cells were counted with a Coulter Counter. The results were plotted on semilogarithmic paper, and the doubling times were calculated from the slopes of the curves. The number of cells in the plateau region of confluent culture was considered to be the maximum cell number able to grow per 5-cm dish.

Tumorigenicity. Cultures of cells were trypsinized, suspended in culture medium, and centrifuged at 800 × g for 5 min. The cell pellet was washed 3 times with PBS and suspended in PBS at the desired cell concentration. Six- to 8-wk-old virgin female BALB/c mice were given injections s.c. in the neck region with 1 to 2 × 10^5 cells in 0.25 ml of PBS. Four- to 6-mo-old virgin female BALB/c mice were given injections with 1 to 2 × 10^6 cells in 20 μl of PBS at three different sites, the mammary fat pad, the thigh muscle, and s.c. in the back.

For injections in the mammary fat pad, a small incision in the flank was made, after which the right posterior abdominal mammary fat pad was exteriorized. The mice were observed weekly for the appearance of tumors. Tumors were examined for morphological type and lungs for metastases using routine histological staining procedures (hematoxylin-eosin).

Antibodies. Rat monoclonal antibodies 117C9, 33B12, T24/40.7, 78B3, JB6, J5E3, GoH3, 50B8, MaB8, 33A10, and 44G3 and polyclonal rabbit antisera to keratins (p-keratin) have been previously described (10). The 50B8 and MaB8 antibodies are directed against different determinants on a M, 170,000 glycoprotein (11). Similarly, the 117C9 and 33B12 antibodies detect different determinants on the Forssman glycolipid hapten (12). T24/40.7 detects Thy-1, and 78B3 is directed against laminin. Mouse monoclonal antibody LE61 was a gift from Dr. B. Lane. The antibody reacts with the keratin filament of simple epithelia and not with stratified squamous epithelia (13).

Immunoperoxidase. Cells were grown on LAB-TEK tissue culture chamber/slides (Miles Scientific, Naperville, IL) and fixed in acetone for 2 to 3 min. The reaction of monoclonal antibodies and polyvalent antisera with the different cell types was tested by an indirect procedure, using second antibodies conjugated to horseradish peroxidase (10). Peroxidase activity was visualized by 3-amino-9-ethyl carbazole and eosin.

Electron Microscopy. Cells were grown in Falcon culture flasks and fixed in situ with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 1 h and postfixed with 1% osmium tetroxide in the same buffer for 2 h. After fixation the cells were scraped off the flasks with a rubber policeman and centrifuged. The pellet was dehydrated and embedded in a mixture of Epon and Araldite. Thin sections were stained with uranylacetate and lead hydroxide. The preparations were examined with a Model EM 300 or EM 301 Philips electron microscope.

Preparation of High-Molecular-Weight DNA. Cells of different cell lines were cultured in Falcon T150 tissue culture flasks. When the cultures became confluent, they were washed in the flask 3 times with PBS and harvested with a rubber policeman. The cells were pelleted at 300 × g for 5 min and suspended in 3 ml of 20 mM Tris-HCl (pH 7.6)-1 mM EDTA-100 mM NaCl. Protease K and sodium dodecyl sulfate were added to concentrations of 200 μg/ml and 1%, respectively. The mixture was incubated at 37°C for 18 h and then extracted twice with phenol/chloroform (1:1) and twice with chloroform at room temperature. Ethanol-precipitated DNA was collected by spooling on a glass rod, dried, and dissolved in 10 ml of Tris-HCl (pH 8.2) and 2 mM EDTA.

Restriction Enzyme Analysis, Electrophoresis of DNA, and Molecular Hybridizations. DNA samples (15 μg) were digested with a 4-fold excess of EcoRI in a volume of 200 μl. The digested chromosomal DNA was ethanol precipitated; dissolved in sample buffer containing 20 mM Tris-HCl (pH 8.2), 10 mM EDTA, 0.2% SDS, and 3% Ficoll, and run in 0.6% agarose gels in buffer containing 0.04 mM Tris-acetate (pH 8.2), 0.02 mM sodium acetate, 0.018 mM NaCl, and 0.002 mM EDTA. DNA was transferred from the gel to nitrocellulose filters according to the method of Southern (14). Recombinant plasmid, PEC-B4, containing the eucaryotic specific sequences of the envelope gene of AKR MuLV (15) and a purified MMTV LTR DNA fragment (16) were used as probes. The DNAs (40 ng) were labeled by nick-translation in reactions containing 50 mM Tris-HCl (pH 7.8), 5 mM MgCl2, 10 mM β-mercaptoethanol, 5 μM each of dGTP and dTTP, 10 μCi each of [3P]dCTP and [3P]-dATP (2 to 3000 Ci/mmol; Amersham International), 10 ng of DNAse I, and 1 unit of Escherichia coli DNA polymerase I in a total volume of 20 μl. After incubation at 15°C for 90 min, unincorporated nucleotides were removed by gel filtration. The specific activity of the resultant probes was 1 to 2 × 10^6 cpm per μg of DNA. Nick-translated probes were denatured at 100°C for 2 min prior to use in hybridization. The nitrocellulose filters with the transferred DNA were incubated with 1 to 2 × 10^6 cpm of radioactive probe in a mixture of 50% formamide, 3× SSC, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2), salmon sperm DNA (200 μg/ml), and Denhardt's buffer (0.02% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone) for 18 h at 41°C. The filters were washed once with 3× SSC-0.1% SDS for 1 h, once with 1× SSC-0.1% SDS for 30 min, and twice with 0.1× SSC-0.1% SDS at 64°C, dried, and exposed to Kodak XAR-5 film.

RESULTS

Cloning and Establishing of Cell Lines

 Cultures derived from a primary adenocarcinoma induced by the RIII strain of MMTV consisted entirely of small, dark polygonal cells. These polygonal cells rapidly induced a tumor upon injection, but stopped growing in vitro after 2 to 3 passages. The tumor was taken in culture, again yielding polygonal cells which had a very limited proliferation capacity. The whole cycle was then repeated, and a cell culture was obtained in which a second cell type appeared: a (large) cuboidal cell. These cuboidal cells could be maintained in culture for many passages, but eventually they became overgrown by elongated cells. They were later used as feeder cells.

The mixed cell population that was derived from the second in vivo-in vitro cycle was reinjected, and the resulting tumor was explanted in culture. This culture contained three dominant morphological types: polygonal; cuboidal; and elongated cells. This mixed cell culture could be passaged 3 times in vitro, after which the individual cell types were separated by cloning in microtitre plates. Cells from five wells were selected for further study: two cuboidal cultures (RAC-2C and RAC-3C); two elongated cultures (RAC-4E and RAC-5E); and one culture containing islands of polygonal cells amid cuboidal cells (RAC-1P). The polygonal cells were further purified from these islands with cloning cylinders and by differential trypsinization. Using this combined procedure we obtained a polygonal cell line, named RAC-10P. A flow chart outlining the different steps used to isolate each of these cell lines is shown in Fig. 1.

Characteristics of Cloned Cell Lines

Polyclonal Cells. At saturation density the cells of the RAC-10P line formed a uniform layer of polygonal cells. When these cells were allowed to grow further, they packed tightly together, developed small vacuoles in their cytoplasm (droplet cells), and spontaneously formed domes (Fig. 2a). Although the polygonal...
cells reached high cell densities, they did not clone in soft agar (Table 1). The RAC-10P line was stable only within a limited number of passages. After several passages, cultures of the RAC-10P line became heterogeneous, and other morphological cell types became apparent. A subclone of the RAC-10P line, termed RAC-11P, appeared to be more stable.

Cuboidal Cells. The cells of the RAC-2C and RAC-3C cell lines formed a flat monolayer of cuboidal cells at saturation density (Fig. 2b). None of these cuboidal cell lines gave rise to polygonal cells. The saturation density of the cuboidal cells was low compared to those of polygonal and elongated cells (Table 1). Cuboidal cells did not produce domes spontaneously nor could they be induced with dexamethasone or dimethyl sulfoxide. From the polygonal cell line RAC-10P, several cuboidal subclones were isolated (Fig. 1). Not all of these subclones were entirely cuboidal. Some of them also contained a small number of cells with a polygonal phenotype indicating a low rate of reversion. The properties of the in vitro-formed cuboidal cells were similar to the ones of the RAC-2C and RAC-3C cuboidal cells.

Table 1  Characteristics of different cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Morphology at saturation density</th>
<th>Saturation density¹</th>
<th>Doubling time (h)</th>
<th>Cloning efficiency in agar²</th>
<th>Domes</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAC-10P</td>
<td>Polygonal</td>
<td>4.1</td>
<td>23</td>
<td>0/10³ ± 1/10³</td>
<td>+</td>
</tr>
<tr>
<td>RAC-3C</td>
<td>Cuboidal</td>
<td>1.4</td>
<td>20</td>
<td>0/10³ ± 1/10³</td>
<td>-</td>
</tr>
<tr>
<td>RAC-31C</td>
<td>Cuboidal</td>
<td>1.1</td>
<td>15</td>
<td>0/10³ ± 1/10³</td>
<td>-</td>
</tr>
<tr>
<td>RAC-34E</td>
<td>Elongated</td>
<td>9.4</td>
<td>15</td>
<td>10 ± 4/10³ ± 2/10³</td>
<td>-</td>
</tr>
<tr>
<td>RAC-5E</td>
<td>Elongated</td>
<td>9</td>
<td>23</td>
<td>50 ± 4/10³ ± 2/10³</td>
<td>-</td>
</tr>
</tbody>
</table>

¹Cells/cm² × 10⁻⁵.  
²Number of colonies/number of cells seeded.

Fig. 1. Isolation of cell lines from a transplanted adenocarcinoma induced by the RIII strain of MMTV in a BALB/c mouse. The morphology of the cell lines is indicated by the last letter: P, polygonal; C, cuboidal; and E, elongated.

Fig. 2. Light micrographs of mouse mammary tumor cell lines. a, droplet cells in a culture of polygonal cells at saturation density with dome (RAC-10P); b, cuboidal cells at saturation density (RAC-3C); c, elongated cells, cloned by soft agar from the cuboidal RAC-3C cell line (RAC-34E); d, elongated cells (RAC-5E).
Upon repeated passage, cuboidal cells generated cells with morphologies intermediate between cuboidal and elongated cells and, ultimately, elongated cells (Fig. 2c). In some instances, foci of elongated cells appeared in cultures of cuboidal lines. The elongated cells could be cloned in soft agar, in contrast to the cuboidal cells (Table 1). Subcloning of cuboidal cells did not prevent them from generating elongated cells.

**Elongated Cells.** The cells of the RAC-4E and RAC-5E lines had an elongated morphology (Fig. 2d). RAC-34E, RAC-313E, and RAC-151E are elongated cell lines, which were isolated *in vitro* from cuboidal cells. Although the different elongated cell lines have comparable morphologies, none of them is completely identical. For instance, the elongated shape of the RAC-34E cells (Fig. 2c) was less pronounced, making them appear to be more epithelioid than the cells of the RAC-5E line (Fig. 2d). There were no major differences in biological properties between the cells of the different elongated cell lines. All elongated cells grew criss-cross, reached high cell densities, and could be cloned in soft agar (Table 1). In contrast to the phenotypic instability of cuboidal and polygonal cells, the elongated cells were stable *in vitro.*

**Ultrastructural Features of the Different Cell Types**

Features of epithelial cells, such as junction formation, microvilli, and B-type particles, were studied by electron microscopy in the different cell types (Table 2).

**Polygonal Cells.** The polygonal cells of the RAC-IP (Fig. 3a) and of the RAC-10P line (Fig. 3b) were covered by abundant microvilli and were joined by well-developed desmosomes. Short bundles of intermediate filaments and A-particles were present in the cytoplasm and B-type particles in the intercellular space. C-type particles were only found in the RAC-1P but not in the RAC-10P cell lines. In the polygonal cell islands of the RAC-IP cultures, cells with myoepithelial features were also found. These cells had rather smooth cell surfaces and large bundles of microfilaments, possibly myofilaments (Fig. 3b), which were oriented parallel to the long axis of the cells and extended from one cell to another. Pinocytotic vesicles were formed on the cell surface of these elongated cells.

**Cuboidal Cells.** The surfaces of the RAC-2C and RAC-3C cells were covered with blunt microvilli with budding particles of the B- and the C-type (Fig. 3c). There were cell junctions of the intermediate type but no desmosomes. The cytoplasm contained intermediate filaments, mainly around the nucleus and intracytoplasmic, as well as intracisternal A-particles. B- and C-type particles were present in the intercellular space (Fig. 3c).

**Elongated Cells.** The elongated cells of the three cell lines (RAC-4E, RAC-5E, and RAC-34E) could be identified as epithelial by the presence of intermediate-type junctions (Fig. 3d). Microvilli were occasionally found in the RAC-4E and RAC-5E cells, but more so in the RAC-34E cells (Fig. 3e).

Although all three cell lines contained intermediate filaments, they were especially abundant in the cytoplasm of the RAC-34E cells (Fig. 3e). Since the cells of this line react with a monoclonal antibody against a keratin component of simple epithelium, the additional intermediate filaments may be composed of keratin. Unlike the elongated cells of the RAC-4E and RAC-5E cell lines, the elongated cells of the RAC-34E cell lines contained A-particles, some intracytoplasmic and many intracisternal, but no B-particles. C-type particles were found in cytoplasmic vacuoles of all elongated cells and the intercellular space (Fig. 3e).

**MMTV and Ecotropic MuLV-DNA Restriction Fragment Analysis in the Original Lines**

To provide evidence on the origin and the cloning of the different cell lines, we examined their DNA for the presence of somatically acquired proviruses of either MMTV or ecotropic MuLV origin.

We first compared the MMTV restriction fragment patterns of the different cell lines. For this purpose, DNAs from the different cell lines were digested with the restriction enzyme EcoRI and blot hybridized to a MMTV LTR probe. EcoRI cleaves the MMTV provirus DNA once, in the center of the genome. Thus, each acquired provirus yields two characteristic fragments, representing the 5' and 3' virus-cell junctions. The size of these junction fragments will be determined by the position of the neighboring EcoRI site in the cellular DNA.

In addition to the MMTV restriction fragments, resulting from the endogenous proviruses, the different cell lines (RAC-10P, RAC-2C, RAC-3C, RAC-4E, and RAC-5E) shared multiple restriction fragments of the same molecular weight (Fig. 4). This indicates that the cells had a common origin. In addition, some cell lines had one or more unique MMTV restriction fragments. The acquisition of these fragments can be explained by new insertions of MMTV proviruses at later stages of tumor development.

Next we examined integrated copies of ecotropic MuLV DNA by rehybridizing the DNA filters with a probe containing the ecotropic specific MuLV sequences of the M, 70,000 glycoprotein gene. The genome of ecotropic MuLV proviruses does not contain an EcoRI site; thus every hybridizing fragment corresponds to a single provirus. Acquired ecotropic MuLV restriction fragments were detected in the polygonal line (RAC-10P) and the two elongated cell lines (RAC-4E and RAC-5E), but not in the two cuboidal lines (RAC-2C and RAC-3C), which contained only a single endogenous provirus. The two elongated cell lines, RAC-4E and RAC-5E, shared all of their acquired ecotropic MuLV proviruses with each other. There was no commonality, however, with the pattern of the acquired ecotropic MuLV proviruses of the parental line (RAC-10P). The detection of distinct acquired ecotropic MuLV restriction fragments confirms our earlier conclusion about the cloning of the cell lines.

**Comparison of the Proviral Restriction Patterns in the in Vitro-generated Subclones**

Various subclones derived from one of these cell lines contained, in addition to the acquired proviruses of the parental

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**Table 2: Ultrastructural features of different cell lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Morphology at saturation density</th>
<th>Microvilli</th>
<th>Desmosomes</th>
<th>Junctions of the intermediate type*</th>
<th>B-particles</th>
<th>C-particles</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAC-10P</td>
<td>Polyvalent</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>RAC-1P</td>
<td>Polyvalent and cuboidal</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RAC-2C</td>
<td>Cuboidal</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RAC-3C</td>
<td>Cuboidal</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RAC-34E</td>
<td>Elongated</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RAC-4E</td>
<td>Elongated</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RAC-5E</td>
<td>Elongated</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* The domains of cellular contact are characterized by a decreased intercellular and an adjacent condensation of electron-dense and fibrillar material in the cytoplasm.

* Elongated cells with smooth surface and polygonal cells with abundant microvilli in islands.

* Only some intracytoplasmic A-particles were found.
Fig. 3. Electron micrographs of mouse mammary tumor cell lines. a, RAC-1P polygonal cells covered with abundant microvilli and joined by desmosomes (D). In the cytoplasm are intermediate filaments (IF) and intracytoplasmic (A) and intracysternal (arrows) A-particles. B-particles (B) are in the intercellular space. × 20,500. b, RAC-1P elongated cells with bundles of microfilaments with focal condensation (thick arrows) running parallel to the long axis of the cells. Desmosomes (D) and pinocytic vesicles (thin arrow). × 22,750. c, RAC-3C cells. The cell surface is covered with blunt microvilli with budding particles (thin arrows). In the cytoplasm, abundant intermediate (IF) and actin (a) filaments. B-particles in the intercellular space (marked area and inset at higher magnification). × 22,750; inset, × 54,600. d, RAC-5E cells. Elongated cells with smooth surface. Indication of junction formation (marked area and inset at higher magnification) with the morphology of the intermediate type, characterized by a decreased intercellular space and an adjacent condensation of electron-dense and fibrillar material in the cytoplasm. × 16,000; inset, × 44,000. e, RAC-34E cells. Elongated cells moderately covered with microvilli. The cytoplasm contains abundant intermediate filaments (marked area and left inset at higher magnification). C-type particles were found in vacuoles (arrows) and in intercellular space (right inset). × 12,300; right inset, × 55,000; left inset, × 41,000.
line, one or more unique proviruses of either MMTV or ecotropic MuLV origin. These unique proviruses were acquired during the propagation of the cell lines in vitro and could be used to confirm the pedigree of the lines. An example is shown in Fig. 5.

The DNAs of the RAC-10P line and several subclones derived from this line were digested with EcoRI, and the MMTV and ecotropic MuLV fragments were compared. Each subclone had the MMTV and ecotropic MuLV pattern of the parent line, RAC-10P. The DNAs of three cuboidal subclones contained additional ecotropic MuLV fragments. Although some of these new fragments were shared between the different subclones, each subclone exhibited a unique combination of fragments. One of the new fragments, however, appeared to be common to all three subclones (Fig. 5, arrowhead). This suggests that the different cuboidal subclones had a common origin.

The observation that elongated cells can be generated without reinserions of either ecotropic MuLV or MMTV proviruses indicates that rearrangements of these proviral DNAs are not required for progression of cells in vitro.

Tumorigenicity and Histology

Various experiments were performed to determine the tumorigenic potential of the polygonal, cuboidal, and elongated cell lines. Table 3 shows the results of one of these experiments in which the tumor-producing ability of polygonal and cuboidal cells was compared with respect to the site of injection.

The polygonal cells of the RAC-10P line rapidly induced tumors at all sites of injections. Tumors developed within 2 to 3 mo after s.c. injections of 1 to 2 x 10^6 cells. At the same cell concentration, the cuboidal cells of the RAC-31C line, a subclone of RAC-3C, were poorly tumorigenic. The cells induced tumors after variable, often long, latent periods (>3 mo), and in some cases no tumors were detected by the end of the observation period (8 mo). Tumors from cuboidal cells were obtained more regularly in the mammary fat pad and in the thigh muscle than s.c. in the back. Analysis of cultures, derived from two different tumors of the RAC-31C line, showed that cuboidal cells formed elongated cells. Combined with the low tumorigenicity and the long latent periods with cuboidal cells, these results strongly suggest that cuboidal cells are not tumorigenic by themselves but that a highly tumorigenic variant (i.e., elongated cells) must be generated in order to give rise to a tumor. Other experiments using the polygonal subclone RAC-11P and various cuboidal subclones isolated from the RAC-10P line also revealed a difference in the tumor-producing ability between polygonal and cuboidal cells. Apparently, the progression of polygonal to cuboidal cells results in a loss of...
tumorigenicity. The different elongated cell lines were all highly tumorigenic. Tumors developed within 1 to 2 mo after s.c. injections of $10^6$ or $10^5$ cells.

The histology of the tumors produced by polygonal, cuboidal, and elongated cells was independent of the site of injection. The polygonal cells produced tumors with a glandular morphology (adenocarcinoma, type B) (Fig. 6a). These tumors had small amounts of stroma and were generally poorly vascularized with necrotic areas. Areas of sarcomatous transformation, locally or more widely distributed, were found in some tumors (Fig. 6b). The tumors produced by cuboidal and elongated cells resembled each other and were sarcoma like (Fig. 6, c and d). Most tumors had invaded muscle tissue and/or skin. None of the cell lines gave macro- or microscopic metastases in the lung.

Distribution of Cell Markers

The distribution of various cell markers was studied in the cells of the different lines by using the immunoperoxidase method. As seen in Table 4, the polygonal cell lines (e.g., RAC-10P and RAC-11P) shared many markers with mammary basal cells. In addition, they reacted with antibodies that are characteristic of epithelial cells (e.g., 33A10). The staining patterns

![Fig. 6. Histological sections, stained with hematoxylin-eosin, of tumors produced by mouse mammary tumor cell lines. a, tumor of RAC-10P cells, showing glandular structures of epithelial tumor cells; b, tumor of RAC-10P cells, showing an area of sarcomatous transformation; c, tumor of RAC-31C cells, showing infiltrative growth of elongated cells in muscle and fat tissue; d, tumor of RAC-5E cells, showing interlacing bundles of elongated cells.](image)

<table>
<thead>
<tr>
<th>Table 4 Detection of cell markers by immunoperoxidase reactions</th>
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</thead>
<tbody>
<tr>
<td>Cell types of mammary gland</td>
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<td></td>
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<tr>
<td>Basal</td>
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<tr>
<td>Myoepithelial</td>
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<tr>
<td>Luminal type I</td>
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<tr>
<td>Luminal type II</td>
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<tr>
<td>Alveolar luminal</td>
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Cell lines in culture

| Polvogonal                  | ++        | +    | -         | ++          | ++           | +   | -            | ++   |
| Cuboidal                    | ++        | +    | -         | -           | +*           | -   | -            | ++*  |
| Elongated                   | +         | +    | -         | -           | -            | +   | -            | -    |

* Staining of various cell types on the basis of intensity of immunoperoxidase reaction over background: -, background; +, weak; +, strong; ++, very strong staining.

* ND, not determined.

* Staining varies between cell clones (see text).
with the various antibodies, in particular with those against antigens on epithelial cells and against keratins (LE61 and p-keratin), were heterogeneous. This heterogeneity was unrelated to the morphological phenotype and may reflect different stages of differentiation.

Cuboidal cell lines (e.g., RAC-2C and RAC-3C) did not stain with p-keratin, but they were positive for LE61. Sublines, however, were usually negative for both LE61 and p-keratin (e.g., RAC-31C and RAC-15C). Thus, the conversion of polyclonal to cuboidal cells was accompanied by the loss of expression of certain keratin genes. Also, the epithelial markers, 33A10 and 44G3, were lost from cuboidal cells. The antibodies 50B8 and MaB8 (luminal markers in ducts, absent in alveoli) showed staining in the cells of the RAC-2C and RAC-3C lines, but other cell lines were negative. Similarly, GoH3 only reacted with the cells of the RAC-2C and RAC-3C lines. The basal cell markers (JB6 and JsE3) remained expressed in all cuboidal cell lines, although in some lines the intensity of staining was lower than in others. In contrast, laminin (78B3) was hardly detectable in the different cuboidal lines.

The elongated cell lines (e.g., RAC-4E and RAC-5E) only reacted with antibodies typical for basal cells of the intact mammary gland. In one case (RAC-34E), reaction was found with LE61, and some cells of this line were also positive with 50B8 and MaB8.

None of these cell lines reacted with mesenchymal markers, such as Thy-1 and Forssman.

Immunocytochemical Staining of Tumors

The reactivity of monoclonal and polyclonal antibodies with tumors from different cell lines was tested on acetone-fixed frozen sections by using the indirect immunoperoxidase method. Tumors induced by the RAC-10P and RAC-11P lines contained glandular structures composed of both basal and epithelial cells. The basal cells reacted with JB6 and JsE3 (Fig. 7a). They differentially expressed the GoH3-defined antigen on the basal sites and were supported by a distinct basement membrane as shown with 78B3, an anti-laminin antibody (Fig. 7b). Basal cells themselves only showed a weak cytoplasmic staining with 78B3. Antibodies 50B8, MaB8, 33A10, and 44G3 reacted strongly with antigens on the apical site of epithelial cells lining the lumen (Fig. 7c). These luminal epithelial cells did not express basal cell markers (JB6 and JsE3). On the other hand, basal cells could express the antigens of the more differentiated epithelial cells, suggesting that they give rise to epithelial cells. The number of basal cells expressing the epithelial markers was variable and different for each cell marker. Monoclonal antibodies 117C9, 33B12, and T24/40.7 stained the connective tissues which surrounded the mammary epithelium.

The reactions with p-keratin were complex. Basal cells were either positive or negative for p-keratin (Fig. 7d), but the majority of basal cells showed staining. In contrast, most of the luminal epithelial cells did not react with p-keratin.

The areas of the tumors showing sarcomatous changes were composed of a flattened cuboidal cell type. These cuboidal cells reacted with markers characteristic for mammary basal cells, but they were usually negative for epithelial cell markers. The sarcomatous areas lacked a 78B3 positive basement membrane.

The tumors induced by cuboidal (RAC-2C, RAC-3C, and RAC-31C) and elongated cells (RAC-4E, and RAC-5E) resembled each other; they lacked glandular structures and consisted of elongated cells. The tumors did not stain with antibodies against keratins (p-keratin and LE61) and the epithelial cell markers 44G3 and 33A10. A weak, often variable, staining was found with 50B8 and MaB8.

The basal cell markers JB6 and JsE3 and the GoH3-defined antigen were demonstrated in all tumors (Fig. 8a). Laminin, detectable with 78B3, delineated the basement membrane of blood vessels (Fig. 8b). Tumor cells were only weakly reactive with 78B3.

![Fig. 7. Immunoperoxidase reactions on mammary tumors induced by RAC-10P cells. Frozen sections were fixed in acetone and incubated with monoclonal antibodies and peroxidase-conjugated second antibodies. a, JB6; b, 78B3; c, 33A10; d, p-keratin.](image-url)
cells, represented in the RAC-1 OP line, can give rise to cuboidal contained cells with distinct morphologies at saturation density: a chemically (7,12-dimethylbenz(a)anthracene) induced mam
aspects in common with the RAMA cell lines developed from genotypic markers.
different steps of the progression pathway by using inserted and then to elongated cells, whereas cuboidal cells of the RAC-
lines derived from a single mouse mammary tumor. These lines
proviral DNA (MMTV and/or ecotropic MuLV DNA) as
DISCUSSION
In this paper we have described the properties of a set of cell lines derived from a single mouse mammary tumor. These lines contained cells with distinct morphologies at saturation density: polygonal; cuboidal; and elongated cells. We have shown that these three cell types are developmentally related. Polygonal cells, represented in the RAC-10P line, can give rise to cuboidal and then to elongated cells, whereas cuboidal cells of the RAC-2C line only give rise to elongated cells. We have confirmed the different steps of the progression pathway by using inserted proviral DNA (MMTV and/or ecotropic MuLV DNA) as genotypic markers.

Relationship to Other Cell Systems. Our system has many aspects in common with the RAMA cell lines developed from a chemically (7,12-dimethylbenz(a)anthracene) induced mammary tumor in the rat, but there are also important differences (6, 9). The cells of the RAC-10P and RAC-11P line resemble the cells of the RAMA-25 line. These cells have retained many characteristics of epithelial cells in that they spontaneously form domes and express many epithelial cell markers. Electronmicroscopic observations revealed the presence of numerous desmosomes and microvilli.

Cuboidal cells, like the ones of the RAC-2C and RAC-3C lines, have not been found in the RAMA-25 cell system. They may not be produced in the RAC-25 cell system, or the instability of these cells does not allow them to be separately cloned. In the RAC cell system, the cuboidal cells are stable only within a limited passage number, and on repeated passage they convert into elongated cells.

The elongated cells of the RAC-4E and RAC-5E lines are morphologically similar to the RAMA-29 cells, derived from the RAC-25 cells. The absence of various epithelial cell markers is also shared by the elongated cells of the two cell systems. There are, nevertheless, also differences between the elongated cells of the RAC and RAMA-25 cell systems. One important difference is that the RAC-29 cells are poorly tumorigenic, whereas the RAC-4E and RAC-5E cells appear to be highly tumorigenic. RAC-29 cells can, however, transform during long-term culturing (17), a property that they share with the cuboidal cells of the RAC-2C and RAC-3C lines.

The conversion of a polygonal to an elongated phenotype, in tissue culture, has also been found in different chemically induced rat mammary tumors (7, 8) and in a virus-induced mouse mammary tumor (4). All of these elongated cell lines were, as our RAC-E cells, highly tumorigenic. In one cell system, RAMA-37, intermediates of polygonal and elongated cells were isolated (8) which have certain features in common with the cuboidal cells of the RAC-2 and RAC-3C lines. These features are: the cell morphology; the presence of little immunoreactive prekeratin; the progression to elongated cells during tissue culturing; and the production of sarcoma-like tumors. The latency periods of the tumors induced by the RAMA-37 cells are, however, considerably shorter compared with those of the cuboidal cells of the RAC system. This difference may be related to the degree of progression the cells have already reached in tissue culture.

Relationship of the Polygonal Cell Type to Cells in the Mammary Gland. We have investigated a possible relationship between the polygonal cells of the RAC-10P and RAC-11P lines and stem cells in the normal mammary gland using a set of cell markers. These cell markers, monoclonal and polyclonal antibodies against cell surface, intermediate filament, and basement membrane proteins, allowed the identification of various cell types in the developing mammary gland (10). In particular, two antibodies (JB6 and JsE3) are important, because they react in end buds and ducts with cells adjacent to the basement membrane. For several reasons it is though that these cells, termed basal cells, are the “stem” cells of the mammary gland. (a) The antigens defined by these antibodies are absent on mature myoepithelial and luminal epithelial cells. (b) Transitional forms have been demonstrated which express both the JB6 and JsE3 antigens and antigens of fully differentiated myoepithelial cells or luminal epithelial cells. (c) We have recently demonstrated that all cells in the very first “anlage” of the mammary gland are positive for the JB6 and JsE3 antibodies. These cells do not yet express epithelial antigens. All of these results strongly suggest that both antibodies react with a pluripotential cell population in the mammary gland.

Other cell markers, which are expressed by the basal cells, are the keratin components, detectable by p-keratin, laminin detectable by 78B3, and the GoH3-defined antigen. The expression of these cell markers, however, is not restricted to basal cells alone, and other cell types within the mammary gland may also be positive.

Within cultures of the RAC-10P and RAC-11P lines, cells were identified which expressed all of the cell markers that are

* J. Daams, T. Sakakura, A. Sonnenberg, and J. Hilgers, unpublished results.

MOUSE MAMMARY TUMOR CELL TYPES

Fig. 8. Immunoperoxidase reaction on mammary tumors induced by RAC-5E cells. Frozen sections were fixed in acetone and incubated with monoclonal antibodies and peroxidase-conjugated second antibodies. a, JsE3; b, 78B3.
associated with basal cells. This suggests a relationship between the polygonal cells in vitro and the basal cells in vivo.

The reaction of the polygonal cells with epithelial cell markers (e.g., 33A10) shows that these cells can undergo processes of differentiation in which they acquire characteristics of epithelial cells. However, they remain positive for basal cell markers. Only in tumors were polygonal cells found that are positive for epithelial markers, but negative for basal cell markers. These cells were lining the lumina of the tumors. Electron-microscopic observations suggest that, in cultures of polygonal cells, in particular in those of the RAC-1P line, differentiation into myoepithelial-like cells also takes place. These myoepithelial-like cells had an elongated shape, smooth surfaces, pinocytotic vesicles, and large bundles of microfilaments with focal densities (18). Myoepithelial-like cells were, however, only occasionally found. Taken together, these results suggest that the polygonal cells are pluripotent capable of differentiating into cells of the two main linkages of the mammary gland: epithelial and myoepithelial cells.

Origin of the Cuboidal Cells. Cuboidal cells originated from the polygonal cells. Within cultures of polygonal cells the cuboidal cells could be recognized by morphology and by lack of reaction with the p-keratin antibodies. They express antigens of basal cells and do not, or only occasionally, express antigens of epithelial cells. In the mammary gland the only two cell types which do not react with the p-keratin antibodies are the luminal type II and alveolar cells.

In contrast to the cuboidal cells in vitro, the luminal type II and alveolar cells in the intact mammary gland are strongly stained by the epithelial cell markers. This indicates that the cuboidal cells do not have a counterpart in the normal mammary gland but probably represent an aberrant differentiation pathway, as a consequence of the neoplastic character of the original cells. In addition, cuboidal cells are negative for the fibroblast markers Thy-1 and Forssman.

Phenotypical analysis and marker studies (reactions with p-keratin) revealed that cultures of cloned cuboidal cells of the RAC-1P line also contained a small number of polygonal cells. This suggests that, under suitable conditions, cuboidal cells can produce polygonal cells. Thus the initial transition from polygonal to cuboidal cells may be reversible. After prolonged culturing and repeated subcloning, this reversibility may be lost, and polygonal cells may convert into “stable” cuboidal cells.

Elongated Cells. A myoepithelial cell differentiation has been proposed to explain the generation of elongated cells in the RAMA-25 system (6). In agreement with this, most of the elongated lines of the RAC cell system were negative for epithelial markers. Nevertheless, a close kinship of elongated cells to myoepithelial cells is not obvious from the distribution of other markers (e.g., JSE3/JB6 and p-keratin). Also, the elongated cells lack distinguishable features of myoepithelial cells in the electron microscope. The distribution of cell markers shows that they are more closely related to basal cells than to myoepithelial cells. Their most conspicuous property is that of malignant transformed cells; they display anchorage-independent growth and lack of contact inhibition. In addition, they are highly tumorigenic. None of these properties is shared by the cuboidal cells which in vitro give rise to elongated cells.

Correlation between the State of Differentiation and Tumorigenicity. We have shown here that the progression from one tumorigenic cell type to another proceeds through a nontumorigenic intermediate. Polygonal cells, themselves giving rise to adenocarcinomas, convert into nontransformed cuboidal cells before the second tumorigenic cell, the elongated cell, arises. The conversion from polygonal to cuboidal cell is accompanied by a loss of epithelial differentiation characteristics. This could indicate that a change in differentiation state is required before transformation to elongated cells can occur. We are currently testing this hypothesis by examining which oncogenes are responsible for the various progression steps and by transfecting known oncogenes into the different cell types.

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