Phenotypic Variance among Cells Isolated from Spontaneous Mouse Mammary Tumors in Primary Suspension Culture

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ABSTRACT

A method is given for selecting epithelial cells directly from primary mammary tumors in Methocel suspension culture. The frequency of colony-forming units in primary tumors was approximately $10^{-4}$. Colonies grew by cell division; formation and growth of colonies was cell density dependent. Five Methocel isolates were established in monolayer culture and characterized. Two were epithelial, evidenced by functional occluding junctions. The other three were not typed in vitro, although they formed carcinomas in vivo. All were subtetraploid by passage 10.

There were variations in ability of the five Methocel isolates to reconstitute in suspension that appeared to be due to the evolution of anchorage-dependent variants during their growth in monolayer culture. These variants could be purified by limiting dilution plating on solid substrates.

The five Methocel isolates and their derivative variants were used to determine correlations between transformation markers and tumorigenicity. Only three Methocel-derived sublines of nine tested, including two recloned in Methocel, were tumorigenic at all when inoculated in two sites of three syngeneic hosts, one athymic. The other six were nontumorigenic. The tumorigenic sublines were less tumorigenic than uncultured cells of parent tumors or parent tumor cells grown in primary monolayer culture. Thus, anchorage-independent growth is not a reliable marker for the tumorigenic mammary phenotype. No correlation was found between two other "contact-related" transformation markers, rapid growth rate and anchorage-independent growth of colonies was cell density dependent. Five Methocel isolates were established in monolayer culture and characterized. Two were epithelial, evidenced by functional occluding junctions. The other three were not typed in vitro, although they formed carcinomas in vivo. All were subtetraploid by passage 10.

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INTRODUCTION

The cellular composition of primary mammary tumors, in terms of the totality of their malignant potentialities and the stability of individual neoplastic phenotypes, is not well known. There is considerable evidence that cells in mammary tumors are not homogeneous in these properties (4, 8, 16, 19, 23, 29). We have sought to isolate and characterize the varieties of cells in mammary tumors.

Cell culture gives a variety of methods for isolating different cell phenotypes directly from tumors (3, 5, 12, 29, 30). Of interest are the 2 general states in culture, anchorage-responsive (monolayer) and anchorage-independent (suspension), the most commonly used methods for mammary tumor cells have relied on anchorage responsiveness (4, 12, 14, 23). The neoplastic phenotype has been partially characterized in cells either in primary short-term cultures or in established lines. In permanent lines, transformation markers have been identified that correlate with tumorigenic growth (4, 5, 10, 11). However, the majority of cells in short-term monolayer cultures display strikingly normal phenotypes, especially in features of contact-mediated growth of colonies was cell density dependent. Five Methocel isolates were established in monolayer culture and characterized. Two were epithelial, evidenced by functional occluding junctions. The other three were not typed in vitro, although they formed carcinomas in vivo. All were subtetraploid by passage 10.
of 1.17% Methocel (4000 cps; Dow-Corning Corp., Midland, Mich.), in Eagle’s 2× MEM (40%), fetal calf serum (20%), sodium pyruvate (1 mm), insulin, and antibiotics as utilized in the agar base. Triple-glass-distilled water was used to bring media to volume. All layers were 0.4 ml in volume.

**Plating Cells in Methocel.** Cells were dissociated from primary tumors or monolayer culture with trypsin (0.05%) and EDTA (0.025%) in phosphate-buffered saline as reported previously (14). Cell viability after dissociation was determined by trypan blue exclusion using a hemocytometer. Packed cells were resuspended in suspension medium [fetal calf serum (20%), 2× MEM (40%), penicillin (100 units), streptomycin (100 μg), and insulin (75 μg/ml)] at a concentration 10-fold in excess of the final concentration desired for plating in Methocel. Cells were added to Methocel (1.3%) at a volume ratio of 1:9 and plated directly on the agar base layer. Additional nutrient Methocel layers were added at 7- to 8-day intervals. All incubations were at 37° in humidified atmosphere of 10% CO₂ (media contain sodium bicarbonate, 4.2 g/liter) in air.

To count viable cells in Methocel, neutral red (1:300, Grand Island Biological Co.) was added in Methocel medium to a final dilution of 1:15,000. At 48 hr after plating, single cells and small aggregates were observed. No aggregates contained more than 10 cells.

The size of colonies in Methocel was determined by measuring their diameters microscopically. The number of cells in colonies was estimated from the colony diameter, assuming that the colony was of uniform cellularity and the diameter of one cell was 12 μm. These parameters were established by histological observation of randomly selected colonies. Cell number was computed from spherical volumes (4/3 πr³). Thus, colonies with 10 to 14, 15 to 19, and >20 cells in diameter would contain 1000 to 2999, 3000 to 7999, and >8000 cells, respectively.

**Colony Isolation.** Colonies were isolated from Methocel suspension with the aid of a micromanipulator and finely drawn glass capillary pipets at X50. Single colonies were placed in 16-mm-diameter Costar wells with 1.5 ml Dulbecco’s modified MEM, with 15% fetal calf serum supplemented with 20% medium conditioned with a primary mouse mammary tumor culture [24 hr conditioning, filtered (0.45 mm)]. Fifty % of the nutrient medium was replaced with fresh medium 3 times per week. Colonies were transferred when they approached monolayering density with the aid of trypsin-EDTA. Once monolayer cultures were established to withstand weekly transfers, conditioned medium was omitted from nutrient media. Cultures were then grown in Eagle’s MEM supplemented with 10% calf serum (Flow Laboratories, Inc., Rockville, Md.) in a closed environment. MEM in Hanks’ balanced salt solution was used for transfer; MEM in Earle’s balanced salt solution was used for routine feeding.

**Other Culture Conditions.** Two colonies, first isolated from Methocel, were cloned after several passages as monolayer cultures. The clones were isolated from plates in an experimental series where the relationship between the number of cells plated and the number of colonies formed was linear.

Five clones were derived by limiting dilution on plastic from MC-1 (passage 14) and MC-2 (passage 15). These clones were isolated in microtiter wells plated at 0.5 cell/well. The clonal origin of the isolates was determined from the linearity of the relationship between number of clones per well on plates seeded between 0.1 and 1.0 cell/well and by microscopic examination of methylene blue-stained sister cultures before growth of cells began.

**Saturation Densities and Doubling Times.** These were determined from growth curves of cells in Leighton tubes (6 sq cm). Determinations were from counts of triplicate cultures. Cell counts were made on Days 1 and 2 after transfer and at 2-day intervals subsequently, immediately preceding medium changes. All cultures were maintained in MEM supplemented with fetal calf serum (10%) and insulin (10 μg/ml) in a humidified atmosphere of 5% CO₂ in air.

**Mammary Tumor Virus Antigens.** Fixed-cell immunofluorescence assays were conducted on cells in monolayer as reported previously (31). Immunoperoxidase assays for MuMTV-positive cells in tumors were conducted using the unlabeled antibody technique of Sternburger et al. (24), modified by Zehr (32). Monospecific antisera to the major envelope antigen (gp52) and the major internal antigen (p28) were obtained from Dr. R. Cardiff and L. Young (University of California, Davis, Calif.). The properties of these sera have been described (26).

**Tumorigenicity Tests for Cultured Cells.** Four hosts were used to determine tumorigenicity of cultured cells: (a) 6-week-old BALB/c males; (b) 6-week-old virgin females; (c) 6-week-old Swiss athymic nude mice; and (d) 3 to 4-week-old BALB/c females. Homozygous (nu+/nu+) Swiss mice were obtained from Life Sciences, Inc. (St. Petersburg, Fla.) under a contract from the Division of Cancer Cause and Prevention, National Cancer Institute. Athymic mice were maintained under pathogen-free conditions in laminar flow (Sta-Clean) hoods.

For transplantation, cells were dissociated from plastic flasks with trypsin-EDTA, counted, concentrated, and resuspended in Dulbecco’s MEM at the desired concentration (1 to 6 x 10⁶) of viable (trypan blue-excluding) cells. Adult mice were inoculated with 0.1 ml s.c. Three 4-week-old BALB/c females were inoculated in epithelium-free mammary fat (7) with a volume of 5 to 10 μl with the aid of a Hamilton syringe and 27-gauge needle.

**RESULTS**

**Growth of Primary Mammary Tumor Cells in Methocel Culture.**

PE. Chart 1 shows the PE of primary tumor cells in primary suspension culture. PE was defined as the percentage of viable (trypan blue-excluding) cells plated that remained viable (neutral red stained) 48 hr after plating in Methocel. PE varied as much as 10- to 20-fold as a direct function of the density of cells plated. For each tumor tested, however, the optimum plating density for maximum PE was within the range of 1 and 8 x 10⁴ cells/sq cm. The PE within the optimal range varied from tumor to tumor. Variance was independent of cell viability at the time of plating (Chart 1). The range in PE at the optimum plating density for cells from 9 primary tumors was between 0.01 and 4%.

Greater than 90% of primary tumor cells were plated as singlets; the remainder were plated as small aggregates, containing between 2 and 10 cells, distributed in a poissonian fashion. The ratio of singlets to aggregates did not vary with plating density. Essentially all (~90%) cell death occurred during the first 48 hr after initiation of suspension cultures, and
it occurred with equal frequency in singlets and aggregates (data not shown).

Chart 1 also illustrates the difference in PE between uncul- 
tured BALB/cfC3H tumor cells and one BALB/cfC3H tumor cell line isolated in Methocel and adapted to long-term mono-

layer culture. A 20% PE was obtained with the established cell line even at the relatively low plating density of 10 cells/sq cm. The behavior of this cell line was typical of other established lines in exhibiting >10% PE and only minor variations in PE as a function of plating density over a wide range of densities.

**Kinetics of Colony Formation.** Chart 2a illustrates typical kinetics of colony formation for cells from primary BALB/cfC3H mammary tumors. Colonies were defined as cellular units which were uniformly stained with neutral red, were a minimum of 10 cell diameters in size, grew progressively, originated from fewer than 10 cells; their formation was inhibited by colchicine.

At a plating density of $8 \times 10^5$ cells/sq cm, colonies were first observed 7 days after plating. The number of colonies increased progressively to a maximum number at 35 days. More prolonged incubation usually resulted in a decrease in colony number. Chart 2a also shows that, while the maximum number of colonies varied directly in relation to an 8-fold difference in the number of cells plated, the maximum number of colonies still occurred at 35 days after plating even at the lowest cell number plated. To demonstrate that the increase in colony number and size was due to cell division, colchicine (0.0004%) was added 24 hr after plating. This treatment completely inhibited colony formation (Chart 2a). Squash preparations of cells in Giemsa-stained intact colonies after 48 hr in the presence of colchicine demonstrated metaphase plates (data not shown).

The lower PE threshold for colony formation in Methocel was between 0.1 and 0.4%. Above a PE of 0.4%, the relationship between the PE and the CFE for cells from BALB/cfC3H tumors was linear over an 8-fold range of doubling dilutions from 1 to $8 \times 10^5$ cells/sq cm (plating density) (Chart 2b). The linear relationship between cell number plated and colony number at 35 days allowed calculation of the number of colony-forming units in primary tumors. Table 1 compares the CFE of 3 cell lines with 2 primary tumor populations in Methocel. When plating efficiency was corrected, essentially every cell in each of the 2 cell lines was capable of growing under our standard conditions of Methocel culture. In contrast, only approximately 1 in 10,000 cells in the 2 primary tumor populations was capable of growth under the same conditions.

**Kinetics of Colony Growth.** Chart 3 illustrates the increase in size of BALB/cfC3H colonies with time of incubation in primary culture. For ease of counting, colonies were grouped...
into 3 categories of size based on overall diameter. Histology
of randomly selected colonies showed that small and large
colonies were of uniform cellularity (see "Materials and Meth-
ods"): small (≥1000 to 2999 cells); medium (≥3000 to 7999
cells); and large (≥8000 cells). The majority of colonies were
small. When 8 × 10^6 cells were plated, small colonies were
first detected 7 days after plating and reached maximum num-
ber at 35 days. Small colonies were the only colonies repro-
ducibly observed at 7 days. At lower densities, even small
colonies were not reproducibly observed until 28 to 35 days.
Colonies of intermediate size were not reproducibly detected
until 21 days after plating at high density. These increased in
number to a maximum at Days 28 to 35. Large colonies were
also first detected 21 days after plating and reached high density. Peak
occurrence of large colonies was also at Days 28 to 35. At
lower density plating, large colonies were observed at 42 days.

Table 1

<table>
<thead>
<tr>
<th>Tumor a, b, c</th>
<th>CFE (%)</th>
<th>PE (%)</th>
<th>Corrected CFE (%)</th>
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<tbody>
<tr>
<td>MC-2u</td>
<td>21 ± 1.2</td>
<td>18 ± 1.2</td>
<td>117 ± 17</td>
</tr>
<tr>
<td>MC-2</td>
<td>32 ± 2.4</td>
<td>39 ± 4.4</td>
<td>82 ± 14</td>
</tr>
<tr>
<td>66C1</td>
<td>34 ± 6.0</td>
<td>28 ± 3.6</td>
<td>120 ± 20</td>
</tr>
<tr>
<td>262</td>
<td>0.00042 ± 0.0003</td>
<td>3.1 ± 0.14</td>
<td>0.013 ± 0.0011</td>
</tr>
<tr>
<td>263</td>
<td>0.000062 ± 0.00004</td>
<td>0.45 ± 0.06</td>
<td>0.014 ± 0.0009</td>
</tr>
</tbody>
</table>

a MC-2u and MC-2 are cell lines isolated from BALB/cfC3H tumors in primary
Methocel culture (see Table 2). 66C1 is a cell line isolated in monolayer culture
from a BALB/cfC3H tumor (8). Specimens 262 and 263 are 2 primary BALB/
cfC3H tumors plated in primary Methocel culture.

Colchicine inhibited the formation of all colonies. Fig. 1, a and
b, illustrates the morphology of large and small colonies, re-
spectively, of primary tumor cells in Methocel culture.

Isolation of Colonies from Methocel

Two colonies, both containing more than 8000 cells, were
isolated from primary Methocel culture of one primary BALB/
cfC3H tumor (Table 2). Both colonies (MC-1 and MC-2) at-

tached and proliferated readily on plastic substrates. The mor-
phology of MC-1 and MC-2 in primary monolayer culture is
shown in Fig. 1, c and d. MC-1 was uniformly epithelioid; MC-2
was epithelioid mixed with fusiform cells. Karyotypes were
determined at 2 passages. At the sixth passage, MC-1 was
tetraploid or subtetraploid (10 of 11 metaphase plates had 70
to 80 chromosomes). At the 11th passage, 119 of 120 cells
were tetraploid or subtetraploid. Thus, the MC-1 karyotype
was stable over 5 early passages. At the eighth passage, MC-
2 was diploid (10 of 11 cells examined). By the tenth passage,
however, only 21 of 126 (26%) metaphase plates were diploid.
The remainder were tetraploid or subtetraploid.

MC-3, MC-4, and MC-5. In a larger experimental series, the
effects of attachment, multiplication, and transfer on establish-
ment of subcultures of Methocel-derived colonies on plastic
was determined. Forty-six isolates were made from primary
Methocel culture of one tumor cell population (Table 2, Tumor 2). Colonies were isolated on Days 15, 27, 40, 50, and 74 after plating in Methocel. Only 61% of the colonies isolated from Methocel attached to plastic. This nonquantitative recovery was, however, probably artifactual due to difficulty in physically separating cells from Methocel, since all those that separated readily attached. Of the colonies that did attach, 79% initiated growth and grew to a monolayer in primary culture. Of those that grew, only 36% survived the first trypsin transfer. Indeed, the sensitivity of cells to trypsin, rather than attachment or growth, appeared to be the major limiting factor in the establishment of subcultures of colonies from Methocel. Cells which grew out from isolated colonies were of 2 morphological types, either epithelioid or fusiform. After trypsin transfer, in secondary passage on plastic (third in vitro passage) cultures were either epithelioid or fusiform. When isolated from Methocel were able to grow in Methocel, we were not due to differences in PE (Table 4).

<table>
<thead>
<tr>
<th>Size of clone</th>
<th>No. of colonies isolated</th>
<th>No. attached</th>
<th>No. attached that grew</th>
<th>No. that grew and transferred</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;3000 cells</td>
<td>35</td>
<td>19 (54)</td>
<td>14 (74)</td>
<td>5 (36)</td>
</tr>
<tr>
<td>≥3000 cells</td>
<td>8</td>
<td>6 (75)</td>
<td>5 (83)</td>
<td>2 (40)</td>
</tr>
<tr>
<td>≥8000 cells</td>
<td>3</td>
<td>3 (100)</td>
<td>3 (100)</td>
<td>1 (33)</td>
</tr>
<tr>
<td>Total</td>
<td>46</td>
<td>28 (61)</td>
<td>22 (79)</td>
<td>8 (36)</td>
</tr>
</tbody>
</table>

Table 3

<table>
<thead>
<tr>
<th>Morphology of transferred cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mixed</th>
<th>Epithelioid</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>&lt;3000 cells</td>
<td>19 (54)</td>
<td>14 (74)</td>
</tr>
<tr>
<td>≥3000 cells</td>
<td>6 (75)</td>
<td>5 (83)</td>
</tr>
<tr>
<td>≥8000 cells</td>
<td>3 (100)</td>
<td>3 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>28 (61)</td>
<td>22 (79)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Morphology in second passage, after one trypsin transfer.
<sup>b</sup> Numbers in parentheses, percentage of colonies.

Growth Characteristics of MC-1 through MC-5. The capability of the 5 Methocel-derived isolates to recolonialize in Methocel after 14 to 17 passages in monolayer culture is shown in Table 4. The CFE of 4 subcultivated isolates was enriched over that observed in corresponding primary culture by a minimum factor of 60 (MC-2) and a maximum factor of 7000 (MC-4). MC-5 did not grow in Methocel over an 8-fold range in plating density. Thus, while isolation of tumor cells in suspension did generally enrich the capability of the isolates to grow in suspension, there were still considerable numbers of cells which did not grow in suspension and considerable variation in that capability among the 5 isolates. The variations in CFE were not due to differences in PE (Table 4).

In an effort to determine why not all cells from colonies isolated from Methocel were able to grow in Methocel, we cloned MC-1 and MC-2 by limiting dilution on plastic and in Methocel to compare the ability of the most prevalent anchor-age-dependent cell to grow in Methocel. The results are shown in Table 5. Cells selected from MC-1 and MC-2 by limiting dilution on plastic (P series) did not grow at all in Methocel under the same conditions of plating and growth by which the original colonies were isolated. Inability of plastic-derived sublines to proliferate in Methocel was not due to low PE's (Table 5). The CFE of cells recloned from MC-1 and MC-2 in Methocel (M series) still varied about 100-fold from 0.3 to 20%. This variance was not due to differences in either passage number or PE (Table 5).

Doubling times and saturation densities were determined for MC-1, MC-2, MC-3, MC-4, and MC-5 as well as for the derivatives of MC-1 and MC-2 recloned in Methocel and on plastic. All cell lines grew exponentially on plastic in the medium used. The doubling times for MC-1 and MC-1m were nearly identical.

Table 4

<table>
<thead>
<tr>
<th>Capabilities of Methocel isolates to reclone in Methocel</th>
</tr>
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<tbody>
<tr>
<td>Clone</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>1C*MT</td>
</tr>
<tr>
<td>MC-1</td>
</tr>
<tr>
<td>MC-2</td>
</tr>
<tr>
<td>2C*MT</td>
</tr>
<tr>
<td>MC-3</td>
</tr>
<tr>
<td>MC-4</td>
</tr>
<tr>
<td>MC-5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Density at which the maximum number of colonies were formed.
<sup>b</sup> 1C*MT and 2C*MT correspond to the initial plating of cells from Tumor 1 and Tumor 2, respectively.
<sup>c</sup> Mean ± S.D.
<sup>d</sup> NT, not tested.
at 17 and 19 hr, respectively. MC-1p cells divided at nearly one-half the rate of MC-1w (30 hr). Population-doubling times for MC-3, MC-4, and MC-5 ranged between 31.2 and 50.4 hr. These data are summarized in Table 6.

After confluence was established, all cultures became stationary, although the density at which saturation was reached was different and in some cases (see Fig. 2b) beyond monolayering. The saturation densities of MC-1w (4.3 \times 10^5/sq cm) and MC-1m (4.2 \times 10^5/sq cm) were virtually identical and not significantly different from MC-1p (3.6 \times 10^5/sq cm). MC-2 and MC-2w also had essentially identical saturation densities. These densities were, however, nearly one-half that of MC-1m (4.2 \times 10^5/sq cm). Also, unlike MC-1p, MC-2p had a substantially lower saturation density than that of the MC-1 Methocel isolates. The 2-fold lower saturation density of MC-2p correlated with a 3-fold slower growth rate than either MC-2 or MC-2w. The saturation densities of MC-3, MC-4, and MC-5 were 3.3, 4.4, and 1.7 \times 10^5/sq cm, respectively.

Expression of MuMTV Antigens. Approximately 90% of cells in the tumors from which Methocel isolates were made reacted with antisera against the major core protein of MuMTV, p28. Only 20 to 35% of cells reacted with anti-gp52 sera (Table 6). MuMTV p28 was also detected in all the Methocel isolates and their derivatives in 80 to 100% of cells (Table 6).

In contrast, the major envelope glycopeptide (gp52) was detected in only a small fraction (5%) of cells from only MC-1 and MC-2, while 85 to 90% of Mmt5/cl cells, isolated from a C3H mammary tumor (19), reacted under these same conditions (Table 6).

Tumorigenicity. We have recently shown that at least 3 hosts, syngeneic male, syngeneic female (mammary fat), and athymic, are needed to assess tumorigenicity of mammary tumor cells (16). Under conditions where uncultured parent tumor cells formed tumors in 100% of inoculated mice with a latent period of 2 weeks, neither MC-1 nor the clones isolated from MC-1 in Methocel or plastic were tumorigenic in any of the 3 test hosts (Table 7).

MC-2 was weakly tumorigenic, forming tumors in 50% athymic nude mice with a latent period of 4 months. Tumors also formed in BALB/c mice after inoculation of 4 \times 10^6 cells s.c. None of 12 mice, however, inoculated with either 2 \times 10^6 or 4 \times 10^6 cells developed tumors after inoculation of MC-2w. Similarly, none of 16 mice developed tumors after inoculation of MC-2w within a period of 6 months.

The low tumorigenicity of MC-1 and MC-2 and their clonal derivatives was not due to some artifact of culture, since cells plated from primary tumors into primary culture exhibited high tumorigenicity; all of 16 mice inoculated with 1 or 2 \times 10^6 cells developed tumors with a latent period of 4 weeks (Table 7). The tumorigenicity of MC-3, MC-4, and MC-5 is shown in Chart 4. Only 2 of these, MC-3 and MC-4, exhibited tumorigenicity in any of 3 test hosts. MC-5 did not form tumors in the subcutaneous of either male BALB/c, female athymic nude mice, or mammary fat of female BALB/c mice at doses of 0.5, 1, 2, and 4 \times 10^6 cells over a period of 18 weeks (only 1 \times 10^6 data are shown). The tumors formed by MC-2, MC-3, and MC-4 were all undifferentiated carcinomas. Histology of one MC-3 tumor is shown in Fig. 2c.

DISCUSSION

The purpose of these studies was to cultivate cells directly from primary mammary tumors in semisolid suspension without an intervening adaptation to serial monolayer subcultivation to compare their neoplastic properties with those of cells isolated directly on solid substrates. Under conditions of suspension culture where 20 to 60% of cells which were previously adapted to long-term culture would survive in Methocel and each sur-

### Table 6

<table>
<thead>
<tr>
<th>Tumorigenicity Classification</th>
<th>Cell line</th>
<th>Cloning efficiency in Methocel (%)</th>
<th>Saturation density (no. of cells x 10^5/sq cm)</th>
<th>Doubling time (hr)</th>
<th>Reactivity to MuMTV antiserum (% of reactive cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumorigenic</td>
<td>MC-4</td>
<td>5.0 ± 1.1c</td>
<td>4.4 ± 0.3</td>
<td>31.2</td>
<td>&lt;1 99</td>
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<tr>
<td></td>
<td>MC-3</td>
<td>0.3 ± 0.04</td>
<td>3.3 ± 0.2</td>
<td>33.6</td>
<td>&lt;1 90</td>
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<td></td>
<td>MC-2</td>
<td>32.0 ± 2.4</td>
<td>2.6 ± 0.2</td>
<td>16.4</td>
<td>5–20 95</td>
</tr>
<tr>
<td>Nontumorigenic</td>
<td>MC-2w</td>
<td>21.0 ± 1.2</td>
<td>2.3 ± 0.2</td>
<td>16.0</td>
<td>&lt;1 85</td>
</tr>
<tr>
<td></td>
<td>MC-1w</td>
<td>2.5 ± 0.28</td>
<td>4.2 ± 0.3</td>
<td>17.2</td>
<td>&lt;1 80</td>
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<tr>
<td></td>
<td>MC-1p</td>
<td>0.98 ± 0.13</td>
<td>4.3 ± 0.2</td>
<td>19.2</td>
<td>5 90</td>
</tr>
<tr>
<td></td>
<td>MC-5</td>
<td>0.00</td>
<td>3.6 ± 0.04</td>
<td>30.0</td>
<td>&lt;1 90</td>
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<tr>
<td></td>
<td>MC-2p</td>
<td>0.00</td>
<td>1.7 ± 0.1</td>
<td>50.4</td>
<td>&lt;1 94</td>
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<tr>
<td></td>
<td>BALB/chMG</td>
<td>0.00</td>
<td>1.5 ± 0.1</td>
<td>52.8</td>
<td>&lt;1 85</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>25.0</td>
<td>18.6</td>
<td>&lt;1 1</td>
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</table>

* Both primary tumors which served as the source of MC-1 to MC-5 contained ~90% (the maximum error in p28 measurements was ±7%) p28-reactive cells and 20 to 35% gp52-reactive cells. Maximum percentages of positive cells in 25 microscopic fields are given. Many fields in positive samples were negative. The C3H MMT5/cl cell line (19) was 85 to 90% reactive with both sera; BALB/c 3T3 was unreactive.

* Tumorigenic ranking 1 to 3 from most to least tumorigenic, was based on tumor-forming potential of cells in 6-week-old BALB/c males and females, 3 to 4 week-old BALB/c females (CFP), and 6-week-old Swiss athymic females.

* Mean ± S.D.

* Nontumorigenicity is based on a tumor-free observation period of 17 weeks in adult BALB/c males and females and 10 weeks in Swiss athymic mice.

* Primary cultures of midpregnant BALB/c mammary epithelial cells (26).
Table 7
Tumorigenicity of cloned mouse mammary tumor cells

<table>
<thead>
<tr>
<th>Clone</th>
<th>Passage no.</th>
<th>Cells $\times 10^6$</th>
<th>Male SQ</th>
<th>Female SQ</th>
<th>Female CFP</th>
<th>Athymic Female SQ</th>
<th>Latent period (wk)</th>
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<tr>
<td>MC-1</td>
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<td>1</td>
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<td>0 (2)</td>
<td>0 (4)</td>
<td>NT</td>
<td>10</td>
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<td></td>
<td>2</td>
<td>0 (4)</td>
<td>NT</td>
<td>0 (2)</td>
<td>NT</td>
<td>26</td>
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<tr>
<td>MC-1</td>
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<td>0 (4)</td>
<td>NT</td>
<td>0 (2)</td>
<td>NT</td>
<td>26</td>
</tr>
<tr>
<td>MC-1m</td>
<td>18</td>
<td>2</td>
<td>0 (4)</td>
<td>0 (2)</td>
<td>0 (2)</td>
<td>NT</td>
<td>26</td>
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<tr>
<td></td>
<td></td>
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<td>NT</td>
<td>0 (2)</td>
<td>NT</td>
<td>26</td>
</tr>
<tr>
<td>MC-1p</td>
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<td>0 (4)</td>
<td>NT</td>
<td>0 (2)</td>
<td>NT</td>
<td>26</td>
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<tr>
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<td></td>
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<td>NT</td>
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<td>100 (4)</td>
<td>NT</td>
<td>100 (4)</td>
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</tr>
</tbody>
</table>

* Male SQ, subcutaneum of BALB/c males; Female SQ, subcutaneum of BALB/c females; Female CFP, cleared mammary fat of BALB/c females; Athymic female SQ, subset of Swiss athymic mice.

Numbers in parentheses, number of animals tested with each cell inoculum.

NT, not tested.

TUMORIGENICITY OF SELECTED TUMOR CELLS

Uncultured Parent Tumor Cells

Chart 4. Tumorigenicity of selected tumor cells. Top. BALB/c males and Swiss athymic female mice were inoculated s.c. with dissociated (uncultured) BALB/c3H mammary tumor cells. Four BALB/c males were inoculated with 4 concentrations of viable (trypan blue-excluding) cells: $\bullet$, 2 x $10^6$ cells; $\times$, 5 x $10^6$ cells; $\bigtriangleup$, 5 x $10^6$ cells; $\triangle$, 1 x $10^6$ cells. Three Swiss athymic females were inoculated with 2 cell concentrations: $\bigcirc$, 2 x $10^6$ cells; $\times$, 1 x $10^6$ cells. Bottom: Four BALB/c males were inoculated each with 1 x $10^6$ viable MC-3, MC-4, and MC-5 cells in passages 7 to 12. Swiss athymic mice (3 to 4 each) were inoculated with 1 x $10^6$ MC-3, MC-4, and MC-5 cells in passages 7 to 12. BALB/c females were inoculated in epithelium-free (cleared) mammary fat with 1 x $10^6$ cells (4 mice for each subline).
vivor would form a colony, only about 1% of primary tumor cells survived, and only about 1 in 10,000 survivors would form a colony. Assuming that cell survivorship was random with respect to ability to proliferate in Methocel and that colonies were derived from less than 10 cells, our data would indicate that anchorage-independent cells exist in primary BALB/cfC3H mammary tumors at a frequency of about $10^{-4}$. These cells would then constitute a minor subpopulation of cells in these tumors. The assumption that death in Methocel was not selective with respect to suspension growth is supported by the parallel linear relationships between colony formation and either cell number plated or cell number surviving 48 hr after plating (Chart 3). Microscopically, we were able to establish that colonies derived from less than 10 cells and the linear relationship between PE and CFE is consistent even with a clonal origin of colonies. These data cannot be considered proof, however, and the frequency of cells with an anchorage-independent phenotype in tumors should be considered accordingly.

Unlike cells adapted to long-term culture, which showed no dependence on density for growth in suspension, the proliferation of primary tumor cells was strictly dependent upon a high plating density. Density dependence for growth of tumor cells is also a hallmark of growth in primary monolayer culture; this property is also lost in long-term monolayer culture (6, 9, 28, 29). It is noteworthy that the optimum density range for monolayer growth in primary culture (9) is the same as that determined in this study for growth in suspension (1 to 8 x $10^{4}$/sq cm). How density regulates growth of mammary cells is a matter of conjecture at present, but the absence of that regulation in long-term lines of mammary cells should be considered when using them as a model to study mechanisms of mammary cell-growth regulation.

Colonies isolated from mammary tumors in Methocel were mixtures of cell types, as evidenced most readily by different morphotypes in primary monolayer culture and by different growth properties in succeeding passages. At least 2 colonies were epithelial, as evidenced by the presence of occluding junctions and the ability to form domes in response to glucocorticoid hormones (13, 21). The cell type of the other 3 colonies studied has yet to be determined. They have not given evidence of occluding junctions but formed carcinomas when inoculated into syngeneic mice. These carcinomas were undifferentiated, similar to transplanted BALB/cfC3H mammary tumors (17). Why differentiated tumors similar to the primary tumors were not encountered is not clear but is probably related to the finding (ref. 16; present work) that primary tumors are composed of large numbers of benign cells that are selected against in long-term culture.

The major population of cells which grew out of 2 Methocel isolates (MC-1 and MC-2) did not proliferate in Methocel under the same conditions in which they were isolated. The number of cells which originated the colonies is critical information for interpreting this and other variations in properties of cells derived from them. Colchicine inhibited colony formation, and mitotic figures were observed in chromosome squash preparations of colonies taken from colchicine-treated cultures; this is evidence that cell division occurred during colony formation. Furthermore, within the optimum density range, the relationship between colony number and plated cell number was linear, which could indicate that the colonies originated from single cells. Clonal origin of primary colonies has not, however, been demonstrated. The data also do not exclude the possibility that other cell types, even nondividing cells, were trapped in growing colonies and plated, and these were selected during monolayer growth. We cannot unequivocally distinguish, therefore, between the emergence of anchorage-dependent variants from anchorage-independent cells and the selection of passenger anchorage-dependent cells. It is tempting to speculate, however, that tumor cells have the capability to change their attachment conditions for growth depending on conditions under which they are grown. Proof of this idea awaits further study with cloned derivatives of primary Methocel isolates.

Isolation of cells on the basis of anchorage-independent growth did not select for highly tumorigenic cells from mammary tumors. An alternative interpretation, that putative anchorage-independent tumorigenic cells were lost in monolayer culture, is unlikely. Highly tumorigenic cells can be readily selected in anchorage-dependent culture (8, 16, 23) suggesting that the time from Methocel isolation to tumorigenicity testing would not necessarily have selected against tumorigenic cells. Indeed, anchorage-independent sublines of cells derived from monolayer culture and grown on plastic were not tumorigenic. Thus, anchorage-independent growth cannot be used as a marker for tumorigenic mammary cells. Indeed, the suspension culture system described here selects epithelial cells from normal mammary glands at nearly the same frequency as from tumors. A lack of correlation between anchorage-independent growth and tumorigenicity has also been suggested by the work of Butel et al. (4) and Hosick et al. (10). Tumorigenicity of cells isolated in Methocel from primary mammary tumors did not correlate with 2 other 'transformation markers', either high saturation density or rapid proliferation rate (Table 7). Both tumorigenic and nontumorigenic lines exhibited both high and low cloning efficiencies in Methocel, high and low saturation densities, and rapid and slow doubling times. Likewise, normal mammary epithelial cells in primary culture could maintain an even higher saturation density than any of the Methocel isolates (Table 7).

There was also no correlation between expression of the 3 transformation markers. Cells with a high cloning efficiency in suspension had a low saturation density; cells with high saturation densities had both rapid and slow doubling times (Table 7). Cells with the highest cloning efficiency (MC-2 and MC-2w) did have the most rapid doubling times; indeed, with one exception (MC-4), all cells which grew in Methocel exhibited much shorter doubling times than did cells that did not grow. MC-4, with a CFE of 5%, had a doubling time between that of the highest and lowest times observed (Table 6).

There also was no correlation between tumorigenicity or expression of transformation markers and expression of MuMTV structural antigens p28 or gp52. A high percentage (>85%) of all clones, both tumorigenic and nontumorigenic, expressed p28 determinants (Table 6). Only 2, one tumorigenic and one not, expressed gp52. Only 5 to 20% of those cells were reactive. Both gp52-expressing lines had a rapid doubling time, but doubling times as rapid were observed in gp52-negative sublines. Similar variations in MuMTV expression have been observed in other mammary tumor cell lines (20). To-

* H. D. Soule, T. Maloney, and C. M. McGrath, unpublished results.
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REFERENCES


Fig. 1. Viable colonies of primary mammary tumor cells in Methocel stained with neutral red: a, a colony with >8000 cells; b, a colony with >3000 cells; c, MC-1 showing epithelioid morphology (multinucleated cells are also shown); d, MC-2, showing morphologic heterogeneity. Both MC-1 and MC-2 were in primary culture on plastic substrates. All are unfixed cultures. × 230.
Fig. 2.  
a, MC-3 in primary culture on plastic showing heterogeneity of cell types; 
b, MC-3 in seventh passage on plastic showing multilayering tendency of fusiform cells; 
c, MC-3 primary tumor, a nonacinar carcinoma; 
d, sixth passage of MC-5 on plastic showing epithelioid cell morphology. Cultures were unfixed and unstained (x 230). Tumor was fixed in Bouin's fixative and stained with hematoxylin and eosin (x 125).
Phenotypic Variance among Cells Isolated from Spontaneous Mouse Mammary Tumors in Primary Suspension Culture

Herbert D. Soule, Terry Maloney and Charles M. McGrath


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