Growth of Rat Mammary Adenocarcinoma Cells in Semisolid Clonogenic Medium Not Correlated with Spontaneous Metastatic Behavior: Heterogeneity in the Metastatic, Antigenic, Enzymatic, and Drug Sensitivity Properties of Cells from Different Sized Colonies

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ABSTRACT

Using rat 13762NF mammary tumor cell clones of varying spontaneous metastatic potentials and biochemical properties and known phenotypic stabilities we studied the relationship between cell colony growth in a clonogenic assay and the biological and biochemical properties of cells derived from different cell colonies. The spontaneous metastatic potential of in vivo or in vitro grown 13762NF tumor cells was not related to their in vitro cloning efficiencies; cells of both low and high metastatic potential formed colonies of various sizes and shapes during 14 days of growth in 0.3% or 0.6% semisolid agarose. A highly metastatic cell clone of relatively low growth potential in agarose was examined further. Individual tumor cell colonies derived from this cell clone were removed from agarose and their properties determined. Cells from small (<100-μm diameter) or large (>500-μm-diameter) agarose colonies had similar self-renewal capacities in agarose and formed variously sized cell colonies when replated in agarose medium. Metastatic potential, drug sensitivity parameters, and expression of a high M, mucin-like glycoprotein antigen and type IV collagenolytic activity known to be associated with spontaneous metastasis of 13762NF tumor cells were dissimilar in cells from different colonies, and these characteristics were independent of original tumor cell colony size in agarose. In contrast, the expression of cell surface proteins of M, < 300,000 were similar among cells derived from different agarose colonies. The data indicate that heterogeneity exists in the ability of 13762NF adenocarcinoma cells of different biochemical and metastatic potentials and drug sensitivities to grow in semisolid agarose. In addition, the cells that grow in agarose to form detectable colonies (>50 cells) are not necessarily those with a high potential of metastasizing spontaneously to distant sites.

INTRODUCTION

A commonly used method to assess the self-renewal potentials of tumor cells and their ability to grow in the presence of antineoplastic drugs or other agents is the clonogenic or stem cell growth assay in semisolid medium (1-3). In this assay the growth of tumor cells in agar or agarose in vitro is thought to be related to the self-renewal properties of the cells in vivo (4). Researchers have been interested in using this assay to predict the sensitivity or resistance of tumor cells to a given therapeutic agent (3, 5), although concerns have been raised that the in vitro growth of tumor cells in semisolid medium may not closely reflect their in vivo growth characteristics, and that cells grown in such assays may not represent the most malignant tumor cell subpopulations growing in vivo (6, 7).

For patients who have solid malignancies that are likely to metastasize, the tumor stem cell or clonogenic assay is one method of assessing tumor cell sensitivity to chemotherapeutic drugs and other agents after initial surgical treatment (8). Since such assays must, for the most part, be conducted with heterogeneous tumor cells obtained from primary neoplasms, their usefulness for predicting the properties of metastatic tumor cells that often exist at low frequencies in primary tumors or tumor cells that have already metastasized remains to be demonstrated.

We have developed an animal tumor model for metastatic breast cancer that can be used to answer certain questions concerning the growth and metastatic potentials of highly malignant mammary tumor cells (9). The 13762NF rat adenocarcinoma system fulfills several criteria that are important in a tumor model for breast cancer, such as its similarity in cell surface (9, 11), antigenic (12), enzymatic (13), and biological (9, 14) properties to breast cancer, and its heterogeneous sensitivities to chemotherapeutic drugs (15), ionizing radiation (16), and hyperthermia (17). Cell clones established from the 13762NF tumor growing at mammary fat pad sites and from spontaneous lymph node and lung metastases have heterogeneous properties (9-17); with prolonged proliferation, they display reproducible phenotypic drift in a number of characteristics, including their metastatic behaviors (10, 18). We used this model of known stability to examine the phenotypes of tumor cells that possess growth potential in a clonogenic semisolid agarose assay.

MATERIALS AND METHODS

Cells and Cell Culture. 13762NF rat mammary tumors were doubly cloned, and the individual cell clones were grown as described previously (9, 10, 14) in AMEM* containing 10% FBS. Cell clones from locally growing tumor (MTC, MTF7) and from spontaneous lung metastases (MTLn2, MTLn3) were used in these studies, and these cell clones were routinely subcultured in dilution ratios of 1 to 50 (MTC and MTF7) or 1 to 150 (MTLn2 and MTLn3) when the tissue culture dishes were ~80% confluent (18). Cell viabilities were determined by trypan blue dye exclusion, and all cell lines were found to be free of Mycoplasma contamination (19).

Animals. Pathogen- and virus-free female Fischer 344 rats (6- to 8-wk-old) were obtained from Charles River Breeding Laboratories (Kingston, NY) in filtered cages and were quarantined for 2 wk. Animals were maintained in a separate colony under the guidelines of the National Research Council, NIH, and The University of Texas System Cancer Center. They were fed Purina 5002 chow and spring water ad libitum.

Biological Assays. Tumor cells were detached from subconfluent cultures using 0.25% trypsin in calcium- and magnesium-free DPBS (18). The suspended cells (>90% viable) were resuspended twice in ice-cold AMEM, and 1 x 10^6 viable single cells were given by s.c. injection in 0.5 ml of medium into the left posterior inguinal mammary fat pad.

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* The abbreviations used are: AMEM, α-modified minimal essential medium; AMEMA, AMEM medium without deoxyribonucleotides or nucleotides; DPBS, Dulbecco's phosphate-buffered saline; FBS, fetal bovine serum; FUdR, 5-fluorodeoxuryridine (Fluoriridine); PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; MAb, monoclonal antibody.
Blocking solution was removed, the cells were washed with 0.05% enzyme-linked immunosorbent assay was utilized. Cells growing in 96-well plates were fixed with cold 0.1% glutaraldehyde for 10 min. The fixed cells were then incubated with 0.2 ml of 0.05% Tween-20, 1% bovine serum albumin in PBS for 1 h at room temperature. This blocking solution was removed, the cells were washed with 0.05% Tween-20 in PBS, and the 40% ammonium sulfate-precipitated fraction of hybridoma HGM1-4.4 was added at a final concentration of 10 μg/ml in PBS-Tween-20 and incubated for 2 h at room temperature. The MAb solution was removed, and the cells were washed with PBS-Tween-20 and then incubated with 50 μg of peroxidase-conjugated goat antimouse IgM (Boehringer Mannheim Biochemicals) diluted 1:1000 in PBS-Tween-20. After a 1-h incubation, the cells were washed 3 times with PBS-Tween and 200 μl of substrate (0.15 mg/ml 2,2′-azino-di[3-ethyl-benzthiazoline sulphonate]) in 0.1 M citrate buffer, pH 4.4, containing 0.5 μl/ml to which 30% H2O2 was added. After 20 min the absorbance at 414 nm was determined using a Titertech Multiscan enzyme-linked immunosorbent assay reader.

Collagenolytic Activity. Degradation of type IV collagen by 13762NF cells was determined as described previously (13). Tumor cells were seeded onto films of [3H]proline-labeled type IV collagen (6 μg, 1000 cpm/μg) and were incubated in 200 μl of AMEM plus 10% FBS. After a 24-h incubation the supernatants were collected and radioactivity was determined. Controls were the same except that tumor cells were omitted. Differential release of radioactivity was completely inhibited by 20 mM EDTA. The release of specific [3H]proline-labeled type IV collagen fragments was assessed by SDS-polyacrylamide gel electrophoresis after a 24-h incubation at 37°C. The characteristic M9, 135,000, 120,000, and 60,000 digestion fragments were found and the appearance of the larger fragments was inhibited by 20 mM EDTA. Tryptsin treatment (1–50 μg/ml for 10 min) of the medium did not result in significant increases in degraded type IV collagen by 13762NF cells, as found previously (13).

FUdR Sensitivities. Drug sensitivity was assessed by a colony-inhibition method described elsewhere (13, 24). FUdR (Hoffman La Roche, Nutley, NJ) was reconstituted in ice-cold AMEMA and diluted before the addition of 5% FBS. The diluted drug was used the same day. Agarose-suspended MTLTS cells were cultured in 60-mm tissue culture dishes containing 5 ml of AMEM plus 5% FBS, and they were incubated overnight in a tissue culture incubator. The medium was aspirated, and 2 ml of prewarmed AMEMA containing 5% FBS and various concentrations of FUdR were added to each dish. After a 4-h incubation at 37°C, the medium was replaced with 5 ml of prewarmed AMEMA plus 5% FBS, and the cells were cultured for 7 days at 37°C. At the end of the incubation, the medium was removed and the cells were fixed with Carnoy's fixative (3:1 v/v methanol:acetic acid) and stained with Giemsa, and the number of cell colonies was determined. A cell colony was scored positive if >50 cells were found (15, 24). Cellular multiplicities and colony survival data were analyzed to calculate surviving fractions. Surviving fractions from replicate experiments were pooled and the dose-response curves determined by computer analysis. Using propagation of error 95% confidence intervals were computed (24).

RESULTS

Cell clones were derived from locally growing 13762NF tumor and its spontaneous lung metastases that have widely differing metastatic potentials and other properties (9–18, 23). The cell clones were analyzed for their clonogenic properties in 0.3 and 0.6% agarose to assess the growth abilities of cells with low and high metastatic potential. We found that in the 13762NF tumor system metastatic potential and agarose clonogenic efficiencies were not related, and this lack of correlation (r = 0.11) existed whether the tumor cells were grown in vivo or in vitro before being assayed (Table 1). Although growth potential in 0.6% agar has been correlated with metastatic potential in fibrosarcoma cells (25), no such relationship was found in the 13762NF system (r = 0.26) (Table 1). Previously, we had found that cell clones derived from the 13762NF tumor and its metastases undergo phenotypic shifts in the metastatic (10, 26) and cell surface (11, 23) properties as well as in their sensitivities to various therapeutic agents (15–18). When the cell clones were examined at the passage numbers where phenotypic shifts occur, we found that their cloning efficiencies in agarose also changed, but the changes in agarose cloning efficiencies and metastatic properties were not related, nor was there any rela-
tionship between agarose cloning efficiencies and growth at s.c. mammary fat pad sites (data not shown).

During growth in agarose the 13762NF cell clones formed cell colonies that varied from <50 μm to more than 700 μm in diameter (Fig. 1). Histograms of the size distributions of a low metastatic (MTC) and a high metastatic (MTLn3) clone indicated that colony size variations were often large and unrelated to metastatic potential (Fig. 2).

To assess the possible relationship of colony size and malignant properties more carefully, we analyzed some of the cell colonies obtained from a low-passage, highly metastatic cell clone, MTLn3. Individual MTLn3 cell colonies were removed from 0.3% agarose after 14 days of growth and expanded for individual analyses of metastatic and other properties. MTLn3 cells are known to be stable during in vitro growth for the number of passages (T3–T8) required to expand the cells for in vitro assay of metastatic potential (10, 18). We found that both small (50–100 μm-diameter) and large (>500 μm-diameter) colonies yielded cells of varying metastatic potential, and when the tumor cells from small or large agarose colonies were grown and replated in 0.3% agarose they reformed cell colonies of various sizes (Table 2 and Fig. 2). Growth of the agarose-derived MTLn3 subclones in vitro or in vivo and reanalysis of their clonogenic potentials in 0.3% agarose also indicated that there was no apparent relationship between spontaneous metastatic potential and growth efficiently in semisolid medium (r = −0.04, r = −0.03) (Table 3).

Since 13762NF cells of varying malignancies express differing amounts of a high M, mucin-like glycoprotein (gp580) in relationship to their spontaneous metastatic potentials (23), we examined the amounts of gp580 using a MAb generated against the purified molecule. As found previously with cloned 13762NF cells, the MTLn3 agarose subclones of highest spontaneous metastatic potential had higher levels of metastasis-associated gp580 (r = 0.9) (Fig. 3). Although differences were found in the expression of the M, 580,000 antigen among MTLn3 agarose subclones, cell surface labeling of lower M, components (M, < 300,000) (26) did not reveal differences after SDS-polyacrylamide gel electrophoresis autoradiography (data not shown). We also examined the expression of metastasis-associated type IV collagenolytic activity and found it to be dissimilar among the MTLn3 agarose subclones. Again the subclones with the highest spontaneous metastatic potentials tended to have higher levels of type IV collagenolytic activity (r = 0.8) (Fig. 4).

Next we examined the 13762NF system to see whether metastatic potential, ability to grow in agarose, and drug sensitivity were related. In examining the agarose-derived subclones of clone MTLn3 cells for their sensitivities to the cell cycle-specific agent FUDR we found that, in general, the FUDR dose-response survival curves of most agarose-derived MTLn3 were similar to that of the MTLn3 parental cells. However, there were some notable differences (Fig. 5). In particular, subclones MTLn3.1, MTLn3.2, and MTLn3.3 had significantly different 50% lethal dose, slope, and y-intercept values (24) from those of the other subclones and the MTLn3 parent.
Table 2 Metastatic potentials of MTLn3 subclones obtained from agarose colony growth assays

<table>
<thead>
<tr>
<th>Clone or subclone (passage no.)</th>
<th>Tumor diameter s.c. (mm ± S.D.)</th>
<th>Initial colony diameter (μm)</th>
<th>Replated average colony diameter (μm ± S.D.)</th>
<th>Spontaneous metastatic potentialb</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTLn3 (T18)</td>
<td>10.1 ± 3.0</td>
<td>&gt;500</td>
<td>200 ± 60</td>
<td>90 (0–250) 9/20 0/20</td>
</tr>
<tr>
<td>MTLn3.1 (T3)</td>
<td>8.7 ± 0.5</td>
<td>50–100</td>
<td>220 ± 70</td>
<td>250 (0–250) 8/8 0/8</td>
</tr>
<tr>
<td>MTLn3.2 (T3)</td>
<td>5.3 ± 1.0</td>
<td>50–100</td>
<td>240 ± 100</td>
<td>0 1/8 0/8</td>
</tr>
<tr>
<td>MTLn3.4 (T3)</td>
<td>9.8 ± 0.9</td>
<td>50–100</td>
<td>200 ± 80</td>
<td>250 (0–250) 8/8 1/8</td>
</tr>
<tr>
<td>MTLn3.5 (T3)</td>
<td>8.7 ± 1.1</td>
<td>50–100</td>
<td>180 ± 60</td>
<td>0 6/8 1/8</td>
</tr>
<tr>
<td>MTLn3.6 (T3)</td>
<td>9.4 ± 1.2</td>
<td>50–100</td>
<td>ND*</td>
<td>0 8/8 0/8</td>
</tr>
<tr>
<td>MTLn3.7 (T3)</td>
<td>8.8 ± 1.1</td>
<td>&gt;500</td>
<td>ND*</td>
<td>0 5/10 0/10</td>
</tr>
<tr>
<td>MTLn3.8 (T3)</td>
<td>9.7 ± 1.2</td>
<td>&gt;500</td>
<td>210 ± 90</td>
<td>0 3/10 0/10</td>
</tr>
<tr>
<td>MTLn3.9 (T3)</td>
<td>10.1 ± 2.1</td>
<td>&gt;500</td>
<td>240 ± 100</td>
<td>0 3/10 0/10</td>
</tr>
<tr>
<td>MTLn3.10 (T3)</td>
<td>9.3 ± 1.5</td>
<td>&gt;500</td>
<td>320 ± 100</td>
<td>0 5/10 1/10</td>
</tr>
<tr>
<td>MTLn3.11 (T3)</td>
<td>9.6 ± 1.6</td>
<td>&gt;500</td>
<td>330 ± 110</td>
<td>250 (0–250) 5/10 1/10</td>
</tr>
</tbody>
</table>

Table 3 Agarose cloning efficiencies of 13762 adenocarcinoma cells obtained from agarose-derived MTLn3 subclones

<table>
<thead>
<tr>
<th>Clone or subclone (passage no.)</th>
<th>Average agarose clone efficiency (% ± SD)</th>
<th>In vivo grown*</th>
<th>In vivo grown†</th>
<th>Spontaneous metastatic potential‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTLn3 (T18)</td>
<td>2.1 ± 0.6</td>
<td>25.1 ± 3.4</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>MTLn3.1 (T3)</td>
<td>16.4 ± 4.7</td>
<td>43.7 ± 3.2</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>MTLn3.2 (T3)</td>
<td>17.3 ± 4.3</td>
<td>30.0 ± 8.6</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>MTLn3.8 (T8)</td>
<td>19.6 ± 7.0</td>
<td>24.6 ± 6.6</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>MTLn3.9 (T8)</td>
<td>6.4 ± 1.3</td>
<td>64.2 ± 4.5</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>MTLn3.10 (T8)</td>
<td>33.8 ± 2.2</td>
<td>20.2 ± 0.3</td>
<td>Low</td>
<td></td>
</tr>
</tbody>
</table>

a Subclones were derived from MTLn3 (T18) cells grown in the mammary fat pads of F344 rats and subcloned in 0.3% agarose. The subclones were then grown in the mammary fat pads of F344 rats, harvested, and analyzed.
b Cells were grown in tissue culture in AMEM plus 10% FBS.
c Subclones were derived from MTLn3 (T18) cells grown in the mammary fat pads of F344 rats, harvested, and analyzed.
d Cells were grown in tissue culture in AMEM plus 10% FBS.

DISCUSSION

The stem cell model of tumor growth predicts that cells capable of growing in semisolid media to form cell colonies larger than 50–100 μm in diameter within 14 days are likely to be cells with extensive self-renewal capacities ("stem" cells), whereas the tumor cells with more restricted growth potential are thought to be "transitional" cells with limited self-renewal properties (4). This suggests that the highly malignant cells that are able to metastasize and grow at distant organ sites are among the clonogenic stem cells. Meyskens et al. (27) recently demonstrated that even small clusters of tumor cells (50- to 60-μm diameter) when removed from agar and replated have the capacity of growing to reform larger cell colonies (up to 200-μm diameter). We also found that 13762NF cells from small colonies have extensive self-renewal capacities and are able to produce larger colonies upon replating in semisolid media. In addition, we could determine the biological properties of tumor cells from small (50- to 100-μm-diameter) or large (>500-μm diameter) tumor cell colonies by s.c. injection of these mammary tumor cells into the fat pads of syngeneic recipients. When this was done, we found a tremendous range in the spontaneous metastatic properties of 13762NF cells of differing tumor cell passages. Among the clonogenic stem cells, the abilities of 13762NF tumor cells to grow in vivo and metastasize appear to be unrelated to their growth potentials in semisolid media.

When various 13762NF cell clones with known metastatic
and other properties were analyzed for their growth properties in 0.3 or 0.6% agarose; no relationship was found between their metastatic and clonogenic potentials. This finding contrasts with those of Cifone and Fidler (25) and Stackpole et al. (28), who reported that tumor cloning efficiencies of murine UV-2237 fibrosarcoma and B16 melanoma in semisolid media were related to their lung-colonization potentials. In the 13762NF tumor system experimental lung-colonization potential and spontaneous metastatic potential to lung are similar (14), but they appear to be unrelated to clonogenic efficiencies or growth rates in agarose. This is not surprising, because the metastatic process is a complex, multistep sequence of events that involves several tumor cell and host properties (29–32), none of which may be similar to the cell characteristics required for growth in semisolid media.

In the 13762NF tumor model, reproducible phenotypic drift has been documented in cell surface components (10, 23) and other properties (9–18), including metastatic behavior (9, 14). This has been shown to be caused by tumor clonal cell divergence that occurs at specific rates (18). 13762NF tumor cells diverge with distinct phenotypic characteristics at differing rates to form heterogeneous cell populations, each with unique properties and sensitivities to various therapeutic agents (18, 24). Phenotypic divergence of cellular properties in other tumor systems has been demonstrated also by clonal cell growth in semisolid media (33–35). The ability of highly malignant tumor cell populations to undergo rapid phenotypic change may be one of the most important and dangerous characteristics of malignant cells (30, 32).

The stem cell cloning or clonogenic assay was developed initially to test human tumor cells for their sensitivities or resistance to various drugs (3, 5, 8, 36–37). Certain practical considerations could, however, limit the usefulness of this assay, an important one being the cellular heterogeneity of malignant tumors with respect to their sensitivities to drugs and other agents (30, 32, 38). Data presented here and elsewhere (15–17, 24) indicate that there is no apparent relationship between the malignant properties of 13762NF tumor cells and their abilities to be affected by any given therapeutic agent. This is not trivial, because cells from primary tumors are used, for the most part, in clonogenic assays, and these tumor cells may not be representative of the few highly malignant cells that are capable of recurring in vivo at metastatic sites. It has been estimated that metastatic cell subpopulations may represent extremely small portions of the total neoplastic cells in malignant tumors (39), and it is known that metastases can form from as few as one blood-borne metastatic cell (40). Moreover, the drug sensitivities of different metastases can be dissimilar. When Von Hoff et al. (5) examined the tumor cell drug sensitivities of primary versus metastases and metastasis versus metastasis in the same patient, poor correlations were obtained. Thus it is not surprising that clonogenic assays do not always predict with a high degree of certainty the outcome of clinical therapy.

Clonogenic assays have been valuable for assessing drug sensitivities as well as resistance of certain types of cancer (37, 41) and in screening of new anticancer agents (42). In breast cancer patients, the use of clonogenic assays to predict therapeutic sensitivities has not been as useful as some other tumors, partly because of difficulties in obtaining adequate samples for analysis. The data presented here suggest that highly metastatic mammary tumors may not be as useful for such analyses because of cellular heterogeneity and the inherent instabilities of highly malignant tumor cell subpopulations. The characteristics of highly malignant neoplasms may allow the growth of only some cells from a tumor in semisolid medium, and when clonal isolates the malignant cells would be expected to proliferate and yield phenotypically diverse progeny in the absence of polyclonal cell-cell and cell-stromal interactions (43).

Note Added in Proof
Substitution of agar or agarose from four additional sources at different concentrations did not change the results.

REFERENCES

HETEROGENEITY OF CLONOGENIC MAMMARY TUMOR CELLS


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