Selection and Characterization in Culture of Mammary Tumor Cells with Distinctive Growth Properties in Vivo

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ABSTRACT

Two epithelial subpopulations with different growth capabilities have been isolated from a mammary tumor cell line. The parent cell line, WAZ-2T, was derived from an adenocarcinoma which developed spontaneously from hyperplastic BALB/c mouse mammary tissue. The subpopulations were isolated using agarose suspension cultures from which anchorage-independent (+SA) and -dependent (−SA) WAZ-2T cells were cultured. Both +SA and −SA sublines grew rapidly in monolayer culture and exhibited stable epithelial morphologies similar to that of the parent line. The cloning efficiency of +SA cells in agarose was 35% while −SA cells did not grow in suspension culture even when plated at high cell density (10^6 cells/25-sq cm flask). Population doubling times in vitro were 17 and 20 hr for +SA and −SA cells, respectively. Production of plasminogen activator was approximately 2-fold greater in +SA cultures as compared to −SA cultures. Both +SA and −SA cells were tumorigenic in BALB/c mice when inoculated s.c. or i.v. However, the latency period for s.c. tumor development was markedly greater for the −SA subline. Similarly, after 4 weeks but not after 7 weeks, significantly more lung nodules developed from i.v. injection of +SA cells than −SA cells. The greater number of +SA lung nodules was not due to increased cell adhesion in the lung. We conclude that established cell lines from mammary tumors can contain widely heterogeneous subpopulations and that these subpopulations provide excellent material for establishing the significance of correlations between in vivo and in vitro behavior.

INTRODUCTION

Tumors are complex mixtures of cells with widely divergent characteristics. It seems probable that both metastasis (10) and tumor progression (16) result from selection of preexisting variant cells within tumors. That mouse mammary tumors in particular are heterogeneous in cell phenotype has been repeatedly shown. For example, the degree of ultrastructural differentiation of epithelial cells in a single mouse mammary tumor ranges from undifferentiated (cycling) cells to completely differentiated (noncycling) cells (23). Furthermore, MMTV-production and antigen expression may vary significantly among clones of mammary tumor cells (17). In yet another type of heterogeneity, mammary tumors of GR mice very probably contain both hormone-dependent and hormone-independent cells since outgrowths from a single tumor can develop into either tumor type (1).

It is important to characterize in detail the various cells which comprise mammary tumors if the biological properties of the integrated lesion are to be understood. It is possible to separate cells which differ in a selected parameter after first dispersing tumor cells into culture. This approach was used by Brides and Kornfeld (3) to select mouse melanoma sublines with different adhesive and spreading properties on plastic substrates. They were then able to correlate these properties with the ability of the cells to produce pulmonary metastases. Similarly, starting with a single mouse mammary tumor, Dexter et al. (7) isolated 4 distinct cell clones, all of which were tumorigenic but which differed significantly in several characteristics in vitro and in vivo.

The primary purpose of this study was to investigate cellular heterogeneity in a mouse mammary tumor cell line using in vitro selection criteria. Cell growth in semisolid medium was chosen as a selection method because a strong correlation has been demonstrated between anchorage independence in culture and tumorigenicity in vivo (11, 21). We found that lack of growth in soft agarose does not select strictly for nontumorigenic cells, but cell populations with markedly different characteristics were nevertheless separated. Growth properties in vivo of the subpopulations were compared and correlated with several key attributes of the cells in culture.

MATERIALS AND METHODS

Cell Culture. The WAZ-2T cell line was derived by Lawrence W. Anderson from a BALB/c mouse mammary adenocarcinoma which developed spontaneously in a hyperplastic alveolar nodule outgrowth [line D1 (15)]. The cells maintained a stable epithelial morphology in culture, and ultrastructural studies revealed the presence of long microvilli, bundles of microfilaments, and prominent desmosomes (6). All cultures were maintained in DME (No. 430-1600; Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% FCS (Flow Laboratories, Inc., Rockville, Md.) and insulin (5 μg/ml; Calbiochem-Behring Corp., La Jolla, Calif.). The WAZ-2T cells were incubated at 37° in a 5% CO2 humidified atmosphere.

agarose Suspension Cultures. The method of agarose suspension culture was essentially as described by Macpherson (14). Agarose (Type 1; Sigma Chemical Co., St. Louis, Mo.) was autoclaved as a 5% aqueous stock solution. Nine ml of 0.5% agarose medium [DME containing 10% FCS, insulin (5 μg/ml), and 0.5% agarose] were pipetted into a 25-sq cm tissue culture flask (No. 3013; Falcon Plastics, Oxnard, Calif.)
The flasks were allowed to equilibrate at 37° in a 5% CO2 feeding, the number of colonies in each flask was determined and allowed to solidify. Six ml of 0.33% agarose medium evaporative loss of water. After a 2-week incubation without feeding, the number of colonies in each flask was determined by counting the number of colonies/100 cells plated in 10 representative areas of each flask.

**Growth Rate.** Replicate 60-mm-diameter culture dishes (No. 3002; Falcon Plastics) were seeded with 2 × 10⁶ cells/dish. At 2-day intervals, cells from 3 dishes were harvested separately and counted with a hemocytometer. Population-doubling times were calculated by the method of Kuchler (13). Saturation densities were defined as the plateau region of the growth curves used to calculate population-doubling times.

**Chromosome Number.** Exponentially growing cultures were incubated with Colcemid (0.5 μg/ml; Grand Island Biological Co.) for 5 hr. Cells were harvested by trypsinization (0.05% trypsin in PBS) and resuspended in 0.75% sodium citrate for 30 min at 30°. Following sedimentation at 1000 rpm for 5 min, the cells were resuspended and fixed in Carnoy’s solution. The fixed cells were spotted on clean slides, allowed to dry, and stained for 45 min with Giemsa. Chromosome spreads of at least 50 nuclei were counted for each WAZ-2T subline.

**Plasminogen Activator Assay.** Fibrinolytic activity was measured by the technique of Chou et al. (5) with slight modifications. Bovine fibrinogen (Type 4; Sigma Chemical Co.) was iodinated after the method of Greenwood et al. (12). ¹²⁵I-labelled fibrinogen was mixed with nonradioactive fibrinogen and dried down in multiwell tissue culture plates (No. 3008; Falcon Plastics) for 4 days at 45° at a concentration of 60 μg/well and approximately 100,000 trypsin-releasable counts/well. Fibrinogen was converted to fibrin by addition of thrombin (1 unit/well; Parke, Davis and Co., Detroit, Mich.) for 3 hr at 37°. Each well was then washed twice with PBS before addition of the assay mixture.

For collection of plasminogen activator samples, cells were plated in 100-mm-diameter tissue culture dishes (No. 3003; Falcon Plastics), allowed to grow to a density of 2 to 3 × 10⁵ cells/sq cm, and then changed to serum-free medium. After a 20-hr incubation, 5-ml aliquots of serum-free medium were removed and frozen at −20°. The assay mixture added to each ¹²⁵I-fibrin-coated well contained 0.5 ml serum-free sample plus porcine plasminogen (5 μg/well; Sigma Chemical Co.). After 16 to 18 hr of incubation at 37°, the fluid in each well was transferred to a 10-× 75-mm culture tube and counted in a Beckman 300 gamma counter.

**Tumorigenicity Studies.** WAZ-2T cells in culture were harvested by trypsinization, and 10⁵ cells were plated in soft agarose suspension culture and allowed to incubate for 2 weeks. A low percentage (<1%) of these cells formed actively growing colonies (diameter, ~250 μm) typical of anchorage-independent cells. Two of the multicellular colonies were removed with a Pasteur pipet and replated in monolayer culture containing DME plus 10% FCS and insulin (5 μg/ml). The resulting cultures were designated +SA. Five to 10 single, nonreplicating cells were also removed from the same suspension culture and replated into monolayer culture (designated −SA). Both +SA and −SA cultures were grown to confluence and split (1:2) until stocks of cells had been stored in liquid nitrogen.

Monolayer cultures of −SA cultures and −SA cells displayed a typical pavement epithelial appearance similar to that of the WAZ-2T parent line (Figs. 1 and 2). Some minor morphological differences between the 2 sublines were noted at confluency, however, +SA cultures contained slightly more multinucleated cells and became vacuolated if left for 1 week at confluency. Fewer multinucleated cells and no vacuolation occurred in −SA cultures. The population-doubling time of −SA cells was slightly greater averaging 20 hr compared to 16 to 17 hr for the +SA subline (Table 1). Saturation densities of both +SA and −SA sublines were 3 × 10⁵ cells/sq cm. Chromosome preparations revealed the presence of typical telocentric mouse chromosomes with approximately 84 to 86 chromosomes/cell for each subline. Both +SA and −SA WAZ-2T sublines were negative for MMTV antigens even when cultured in the presence of insulin (5 μg/ml) and hydrocortisone (5 μg/ml).
To test the stability of the anchorage-independent (+SA) and anchoragedependent (−SA) phenotypes, the 2 isolated sublines were tested repeatedly for growth in suspension culture. Colony-forming ability of +SA cells was 35%, and colony diameters ranged up to 575 μm. Typical morphology of +SA colonies is shown in Fig. 3. In contrast, −SA cells consistently lacked colony-forming ability, no identifiable colonies/10^5 cells seeded arose after 2 weeks of incubation. Furthermore, colony-forming efficiency of +SA and −SA cells did not change with continuous passage in monolayer culture.

To determine if anchorage independence and enhanced plasminogen activator production were associated (19), release of plasminogen activator extracellularly by +SA and −SA confluent monolayers was compared. After a 20-hr incubation in serum-free medium, +SA cell monolayers released approximately twice the amount of enzymatic activity as compared to −SA cells (Table 2). Lysis of 125I-fibrin was dependent on the presence of plasminogen, indicating action of a plasminogen activator.

In Vivo Characteristics of +SA and −SA WAZ-2T Cells

Growth s.c. of Injected +SA and −SA Cells. The growth potential of +SA and −SA cells in vivo was tested by inoculation of 10^6 cells s.c. into syngeneic female BALB/c mice. Within 5 weeks, +SA cells produced tumors in 11 of 11 mice with a latency period (time for palpable tumor formation) of 9 days (Table 2). The mean +SA tumor weight was 2.37 g. In contrast, −SA cells by 5 weeks postinoculation produced only small tumors (mean weight, 0.33 g) in 5 of 10 mice with an average latency time of 24 days. The remaining 5 −SA mice did not develop palpable tumors until Day 40. In a separate experiment, it was found that large −SA tumors (weight, 2 to 3 g) were present by 50 to 60 days. Thus, both sublines produced s.c. tumors, but the latency period was markedly increased for −SA cells.

Histological examination of s.c. +SA and −SA tumors demonstrated that both sublines produced type B adenocarcinomas according to the classification of Dunn (8). +SA tumors were poorly differentiated with scattered glandular elements and extensive areas of intermingled epithelial and connective tissue cells (Fig. 4). −SA tumors, in contrast, were composed of well-defined epithelial cords surrounded by fibrous stroma (Fig. 5). Necrosis occurred in both +SA and −SA tumors; occasionally, invasion into adjacent muscle tissue was observed. Inflammatory cells were more common in +SA tumors.

Enzymatically dissociated tumor cells of +SA and −SA origin (passaged once in vivo) were recultured in primary monolayer culture and subsequently tested for growth in semi-agarose medium. −SA cells derived from a bona fide epithelial −SA tumor failed to produce colonies in suspension culture when plated at 10^6 cells/flask, indicating stability of the anchorage-dependent (−SA) phenotype. Clearly, s.c. tumor formation did not enrich for a small population of malignant anchorage-independent cells present in the −SA subline. As expected, recultured +SA tumor cells produced about 30 to 40 colonies/100 cells plated in suspension culture after 2 weeks of incubation.

Pulmonary Growth of i.v. Injected +SA or −SA Cells. The ability of +SA and −SA cells to produce metastatic nodules in the lung was examined by inoculation of 10^5 or 5 × 10^5 cells into the lateral tail vein of syngeneic BALB/c mice. Four weeks after injection of 10^5 +SA cells, tumor nodules were readily discernible (Table 3, approximately 4/nodule set) with a diameter of 0.5 to 1.0 mm (Fig. 6). In contrast, injection of 5 × 10^5 −SA cells rarely produced macroscopic nodules within 4 weeks. Most −SA nodules were identifiable only in histological sections (Fig. 7). However, if −SA mice were sacrificed after 7
weeks, large nodules up to 5 mm in diameter were present in their lungs (Fig. 8). Injection of $5 \times 10^5$ cells i.v. showed a similar difference in time of appearance of large nodules when comparing +SA and −SA sublines (Figs. 9 and 10). Therefore, both +SA and −SA cells were capable of survival in the lung, but the extent of growth after 4 weeks was much greater for +SA cells.

The difference between the sublines may reflect differential retention of cells in the lungs. +SA and −SA cells in culture were labeled with [25]IdUrd and inoculated i.v. into syngeneic female BALB/c mice. The retention of +SA and −SA cells in the lungs over the initial 72-hr postinoculation period is presented in Table 4. The number of cells initially retained was virtually 100% for both +SA and −SA cells. However, the survival of +SA cells in the lungs was significantly lower than that of −SA cells by 60 min. This survival difference persisted throughout the remaining 72-hr test period. Thus, +SA cells, while “sticking” to the lung vasculature to the same extent as −SA cells, were cleared more rapidly.

DISCUSSION

Experiments on 5 cell lines derived from mouse mammary tumors by Butel et al. (4) have shown that anchorage-indep-endent mammary cells do not invariably produce tumors in vivo. Specifically, 4 clones which readily grew in agarose were not tumorigenic in syngeneic mice. Interestingly, only one clone of 17 studied by Butel et al. did not grow in agarose, and these cells were nontumorigenic in mice. Thus, one of the questions we wished to address was whether mammary cells which do not grow in semisolid medium could ever be tumorigenic in vivo. In our study, we found that tumors could develop from −SA cells. Therefore, anchorage independence of growth and in vivo tumorigencity are not always linked in mammary tumor cells. We have further shown that epithelial cells derived directly from −SA tumors still lack the ability to grow in agarose culture. This experiment eliminates the possibility that either a very small subpopulation of anchorage-independent cells in the −SA population or a spontaneous phenotypic change of −SA cells was responsible for tumor formation.

Comparisons of +SA and −SA cells in vivo revealed a significant difference in latency of tumor formation as defined by development of palpable tumors. The difference in tumor latency time was best illustrated following i.v. inoculation of $5 \times 10^5$ subline cells. Four weeks after inoculation, the average tumor nodule count/lung set was 191 +SA nodules as op-posed to 16 −SA nodules. In sharp contrast to these results, by 7 weeks, both +SA- and −SA-inoculated animals contained a large number of tumor nodules (see Table 3 and Fig. 10). A similar delay of −SA tumor development was found after s.c. injection.

One possible factor involved in the difference in +SA and −SA tumor development could be cell cycle time. Our limited results on this variable indicate that +SA cells in vitro have a shorter population doubling time than do −SA cells. Braun-schweiger et al. (2) have studied growth characteristics of a large number of spontaneous mammary tumors using in vitro and in vivo techniques. They have shown that rapidly growing tumors have shorter cell cycle times and larger growth fractions than do more slowly growing mammary tumors. A similar finding was made by Pavelic et al. (18) using 2 transplantable mouse mammary tumors with different growth rates in vivo. What is not clear as yet with the +SA−−SA system is whether the rate of tumor growth after tumor formation differs for the 2 sublines.

+SA and −SA tumors are classified as type B adenocarcinomas [Dunn classification (6)]. −SA tumors closely resemble tumors developed from WAZ-2T parental cells with well-developed cords of tumor cells surrounded by connective tissue. +SA tumors contain densely packed tumor cells with a few well-defined cords of tumor cells. Pavelic et al. (18) have noted a similar difference between rapidly and slowly growing transplantable mammary tumor lines. Therefore, the rate of tumor growth in vivo may be an important factor in the histological differences between +SA and −SA tumors. No sarcomatous modification of tumor histology was observed with WAZ-2T cells as has been previously reported with other cultured mammary tumor cells (20).

Development of tumor nodules after i.v. injection of tumor cells can be influenced by initial cell adhesion and subsequent rate of cell clearing in lung and other organ tissue (9). The injection of [25]IdUrd-labeled +SA and −SA WAZ-2T cells demonstrated an initial arrest of 100% of the cells of both sublines in the lung vasculature. Therefore, early differences in lung nodule counts between +SA and −SA cells are not due to differences in total number of initially adhering cells. However, over the subsequent 72 hr, +SA cells were cleared more readily than were −SA cells resulting in a sharp difference in arrested cell number on Day 3. As previously noted, over a 7-week period, a large number of tumor nodules will develop in +SA- and −SA-injected mice. Thus, the cell reten-tion data would suggest that a greater proportion of +SA cells than −SA cells retained at 72 hr formed tumor nodules in the lung. Whether the number of cells retained somehow alters the time required for nodule development is unclear. However, s.c. inoculations provide evidence that the latency difference between the 2 sublines is an intrinsic property of the tumor cells.

Anchorage independence has often been linked to a rise in plasminogen activator synthesis (19). Our results demonstrate a similar association in WAZ-2T cells. It should be noted, however, that even the −SA subline actually produces rather copious quantities of plasminogen activator and that the proposed increase in activator synthesis by +SA cells can be justified only by comparison with −SA cells.

We have shown that a widely used in vitro criterion for tumorigenocity may be used to select different subpopulations of tumorigenic cells for further study in vivo. The successful isolation and passage of WAZ-2T +SA and −SA sublines indicate that the selected phenotypes are stable. By using such closely related but functionally distinct tumorigenic cell popula-tions, we hope to determine the role of selected cell properties during tumorigenesis in vivo.

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REFERENCES


Fig. 1. WAZ-2T +SA confluent monolayer culture. Bar, 50 μm. H & E, x 420.
Fig. 2. WAZ-2T -SA confluent monolayer culture. Bar, 50 μm. H & E, x 420.
Fig. 3. +SA colonies in suspension culture after 3 weeks of incubation. Bar, 100 μm. Phase contrast, x 190.
Fig. 4. Section of a +SA adenocarcinoma 4 weeks after s.c. injection of 10⁶ cells. Epithelial cells are intermingled with connective tissue elements. Bar, 100 μm. H & E, x 275.
Fig. 5. Section of a +SA adenocarcinoma 8 weeks after s.c. injection of $10^6$ cells. Note cords of epithelial cells surrounded by stroma. Bar, 100 μm. H & E, × 275.

Fig. 6. +SA tumor lung nodule 4 weeks after i.v. injection of $10^6$ cells. Bar, 100 μm. H & E, × 275.

Fig. 7. +SA tumor lung nodule 4 weeks after i.v. injection of $10^6$ cells. Bar, 100 μm. H & E, × 275.

Fig. 8. +SA tumor lung nodule 7 weeks after i.v. injection of $10^6$ cells. Bar, 100 μm. H & E, × 275.
Fig. 9. +SA and −SA pulmonary lobes 4 weeks after i.v. injection of $5 \times 10^5$ cells. Note absence of visible tumor nodules on −SA pulmonary lobes. Bar, 2 mm. $\times$ 3.75.

Fig. 10. +SA and −SA pulmonary lobes 7 weeks after i.v. injection of $5 \times 10^5$ cells. Bar, 2 mm. $\times$ 3.75.
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