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Relationship of Cell Growth Behavior *in Vitro* to Tumorigenicity in Athymic Nude Mice[1]

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SUMMARY

The serum requirements, anchorage requirements, saturation densities, and contact inhibition responses of a variety of mammalian cell lines were determined under uniform conditions. The serum requirement of both transformed and normal cells was a sensitive function of initial plating density. Cloning efficiency on irradiated mouse monolayers was found to be an invalid indicator of contact inhibition of growth, since most cell lines that failed to form visible colonies on cell monolayers nonetheless proliferated on these monolayers. When normal and neoplastic cells from a variety of sources were examined, none of the growth parameters that serve to define the transformed state *in vitro* correlated consistently with cellular tumorigenicity in athymic nude mice. It is concluded that the most reliable and physiologically meaningful assay for malignant transformation is, at present, cellular tumorigenicity in athymic nude mice.

INTRODUCTION

Normal animal cells may be transformed into cancer cells following exposure to carcinogens *in vivo* or *in vitro*. When transformation occurs, growth behavior *in vitro* is typically altered. Transformed tissue culture cells may display a reduced serum requirement for growth (4, 17), loss of anchorage requirement (20, 21), loss of contact inhibition of growth (22), and/or increased saturation density (23) relative to their normal counterparts. Investigators from different laboratories have sought to determine which, if any, of these modifications to growth behavior *in vitro* is correlated with tumor formation in animals (1, 5, 8, 15, 16, 25). Aaronson and Todaro (1) determined that a high saturation density correlated best with tumorigenicity. Weiss *et al.* (25) reported that loss of contact inhibition of growth and locomotion was the best *in vitro* indicator of tumorigenic potential. Still other investigations have concluded that only anchorage requirement *in vitro* correlates with tumorigenicity (5, 8).

For both theoretical and practical considerations, it would be useful to understand the relationship between growth behavior of tissue culture cell lines and growth control of normal animal tissues *in vivo*. A review of previous attempts to determine this relationship suggested to us that 1 source of the discrepancies was the assay for tumorigenicity. After prolonged growth in the laboratory, animal cell lines can acquire new or modified surface antigens as a result of viral infections, mutations, or epigenetic changes. These altered surface antigens can mask the tumorigenicity, even of cell lines derived from inbred animal hosts by provoking an immune response. Measurements of cellular tumorigenicity are probably least ambiguous when conducted in immunologically suppressed animals.

Additionally, many, if not most, studies on transformation and comparative tumorigenicity have used secondary embryonic cell strains and cell lines such as 3T3 and BHK as the "normal" controls. The bulk of human neoplasms are of epithelial origin. Therefore, it would be desirable to conduct comparative tumorigenicity studies with cell lines of epithelial origin to determine whether principles established with embryonic cell strains and "fibroblast-like" cell lines can be extended.

In a previous communication (18), our laboratory presented evidence that the congenitally athymic nude mouse is a reliable host for assessing the tumorigenic potential of both animal and human cell lines. The tumorigenic potential of fibroblast-like and epithelioid cell lines derived from both normal and neoplastic tissue was determined. To determine the extent to which growth-regulatory behavior commonly observed *in vitro* such as serum requirement, anchorage requirement, density-dependent growth inhibition, and contact inhibition of growth are relevant to tumor formation in animals, we have correlated these properties with the tumorigenic potential in nude mice. The results of these studies are presented below.

MATERIALS AND METHODS

Cell Culture. Tissue culture cell stocks were maintained in DME-HSFBS.[4] All culture media, balanced salt solutions, and animal sera were obtained from the Grand Island Biological Co., Grand Island, N. Y. The origins and biological

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[4] The abbreviations used are: DME-HSFBS, Dulbecco-Vogt modified Eagle's medium supplemented with 12.5% horse serum and 2.5% fetal bovine serum; DME, Dulbecco-Vogt modified Eagle's medium; FBS, fetal bovine serum; PBS, calcium- and magnesium-free Dulbecco's phosphate-buffered saline.

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properties of the cell lines and cell strains used in this study have been described in detail in a previous report (18). All cell lines were screened for *Mycoplasma* contamination before use by autoradiography and were negative for infection by this test.

Test for Tumorigenicity. The procedures for, and results of, tumorigenicity testing in nude mice have been described in detail in a previous report (18). In brief, congenitally athymic nude mice with the BALB/c genetic background were maintained in isolated facilities under sanitary, but not germ-free, conditions. Nude mice in our colony have a life expectancy of up to 2 years. Between 1 and 2 million cells of each line were inoculated s.c. into the scapular region. With an inoculum of this size, most tumorigenic lines generated tumors between 7 and 21 days postinoculation; however, all animals were observed for at least 4 months for signs of tumor formation. Those cell lines that failed to generate tumors with an inoculum of 1 to 2 $\times$ 10^6 cells were retested in the same fashion with inocula of up to 10^7 cells. All tumors of human origin and randomly selected tumors of animal origin were examined histologically. In every case, the nodules proved to be authentic neoplasms rather than inflammations or infections.

Determination of Serum Requirement. Comparative growth rate measurements in DME supplemented with 10 or 1% FBS were performed using a single lot of FBS obtained from the Grand Island Biological Co.

Between 1 and 2 $\times$ 10^4 cells were inoculated as single-cell suspensions into 60-mm Nunc tissue culture dishes (Vanguard International, Red Bank, N. J.). The growth medium used for the initial plating was DME-HSFBS. Twenty-four hr after inoculation into this serum-rich medium, the medium from 1 sample plate was removed by aspiration. The cells adherent to the culture dish were washed once with PBS and then exposed to 0.01% trypsin in PBS for 10 min. The number of adherent cells released following trypsin treatment was determined with the Coulter particle counter. Growth rate measurements were initiated only when the culture dishes contained between 1 and 2 $\times$ 10^4 adherent cells; cultures containing fewer than 1 $\times$ 10^4 adherent cells were incubated in DME-HSFBS and counted daily until the plates contained between 1 to 2 $\times$ 10^4 cells. Cultures that initially contained in excess of 2 $\times$ 10^4 adherent cells per plate were not used for growth rate measurements.

To initiate a growth rate measurement, DME-HSFBS was removed from all of the culture dishes by aspiration, and all cultures were washed twice with sterile PBS. One-half of the dishes received DME supplemented with 10% FBS and the others received DME with 1% FBS. On alternate days, cells from sample plates were resuspended by brief exposure to trypsin and counted with the Coulter particle counter. The culture medium was changed on alternate days. The population-doubling time of the cells in 10% FBS and in 1% serum was determined. The serum requirement of a cell line was expressed as the ratio of the doubling time in 10% serum to the doubling time in 1% serum. Thus, for cells with a low serum requirement, this index approaches unity and for cells with a high serum requirement, the ratio will be close to zero.

Determination of Anchorage Requirement. Cells were plated in Methocel suspension essentially as described by Risser and Pollack (13); Nunc culture dishes (60 mm diameter) were covered with DME-HSFBS in 0.9% agar as described by these authors. The culture medium was DME-HSFBS supplemented with 1.2% Methocel (4000 cps; from the Dow Chemical Co., Midland, Mich.). Duplicate agar-coated dishes were inoculated with 10^2, 10^3, or 10^4 cells that had been suspended in 4.0 ml of Methocel medium. At weekly intervals for 3 weeks, 3 ml of Methocel medium were added per culture dish. After 4 weeks of total incubation, the number of clones per dish greater than 0.2 mm was determined; only those cultures containing between 20 and 200 such colonies were scored. In parallel with the growth of cell clones in Methocel, duplicate cultures of 10^2, 10^3, or 10^4 cells were inoculated into 60-mm Nunc tissue culture dishes with DME-HSFBS as the growth medium. The medium in these cultures was changed once after 7 days. At 14 days, the medium was aspirated from these cultures. The cells were fixed with 10% formalin and stained with crystal violet. Stained colonies were counted in those culture dishes containing between 20 and 200 clones. The anchorage requirement of individual cell lines was expressed as the ratio of colonies formed in Methocel medium to colonies formed on bare culture dishes under these conditions.

Determination of Saturation Density. The saturation density of individual cell lines was determined in conjunction with the growth rate measurements described above. The number of cells per sq cm of culture dish surface was calculated from that point in the growth curve with 10% FBS, where the increase in cell number per plate stabilized or, as with some cell lines, began to decrease as cells detached from the dish.

Determination of Contact Inhibition. Normal mouse embryo cells were grown to confluent monolayers on plastic culture dishes and then exposed to 3000 rads of ^{60}Co irradiation. Single-cell suspensions of cell lines to be tested for contact inhibition of growth were prepared in PBS. Suspensions of 100 and 1000 test cells were inoculated in duplicate into 60-mm Nunc culture dishes and into 60-mm Nunc dishes covered with monolayers of irradiated mouse fibroblasts. All cultures were incubated 2 weeks in DME-HSFBS with 1 change of medium at 7 days. After 2 weeks, all of the cell cultures were fixed with 10% formalin and stained with crystal violet. Stained colonies on the bare plastic dishes and dense cell foci on the irradiated monolayer cultures were counted. Only those cultures containing between 20 and 200 clones or foci were scored. The contact inhibition response of a cell line is expressed as the ratio of foci formed on irradiated mouse fibroblast monolayers to colonies formed on plastic culture dishes.

Several cell lines failed to form visible foci on mouse monolayers and these were tested for growth in another way. Cells were exposed to trypsin and resuspended in growth medium at a density of approximately 4 $\times$ 10^5 cells/ml. The number of colony-forming cells in suspension at time 0 was determined by serially diluting the cells, plating into culture dishes, and incubating at 37° with biweekly medium changes until visible colonies appeared. One ml of the cell suspensions was plated onto duplicate 60-mm culture dishes with and without irradiated monolayers of mouse fibroblasts. The plated cells were incubated for 4 days with a medium change at 2 days. After 4 days of

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incubation, the cultures were harvested by trypsinization. The culture medium from each dish was saved and added back to the trypsinized cells to ensure that any viable, floating cells would be counted. The harvested cells were serially diluted, plated, and incubated at 37° with biweekly medium changes until colonies had appeared. Control experiments demonstrated that irradiated mouse fibroblasts alone would not form colonies and would not interfere with colony formation by viable cells. The ratio of colony-forming units per plate after 4 days of growth to colony-forming units seeded initially was used as an index of contact inhibition of growth.

RESULTS

The generation time of cells grown in medium supplemented with 1% FBS proved to be a sensitive function of the number of cells plated/dish. The effect of cell density and serum concentration on the growth of the VA2-8-aza-G^r cell line is depicted in Table 1. As can be seen, population-doubling time with this cell line responded in a nongraded fashion to initial plating density. When the cell density was less than 5×10^3 cells/sq cm (10^5 cells/60-mm plate), growth did not occur in DME with 1% FBS. When the density of VA2 cells was increased by a factor of 5, logarithmic growth occurred with a doubling time actually shorter than that observed in DME with 10% FBS. With the particular lot of serum used in these experiments, 3T3 cells proliferated when 5×10^4 cells were initially presented in 60-mm dishes but not when 10^4 cells were initially adherent. Since the 3T3 cell line is a widely accepted standard of comparison in transformation studies, we conducted all serum requirement determinations using a starting density of 1 to 2 $\times 10^4$ cells/60-mm culture dish.

Table 1
Effect of initial density on growth of VA2-8-aza-G^r cells in low- and high-serum-containing medium

Growth medium	Population-doubling time (hr) when the initial cell population/60-mm culture dish is			
	5×10^5	10^5	2×10^4	4×10^3
DME + 10% FBS	58	47	48	50
DME + 1% FBS	40	41	∞	∞

Table 2 ranks the various cell lines tested in order of increasing serum requirement for growth. No absolute correlation is seen between tumorigenicity of the cells in nude mice and serum requirement. The BRLC cell line exhibited a low serum dependence and did not form tumors in nude mice. The tumorigenic RPMI-2650 line would not grow in either 10 or 1% DME-FBS at an initial cell density of 10^4/plate.

To determine whether tumor formation selects for variant cells with a reduced serum requirement for growth, a tumor formed from the injection of B-16 melanoma cells was excised from the nude mouse and plated into culture. As shown in Table 2, the serum requirement of the tumor derived cells (B-16T) is not significantly lower than that of the cells originally injected into the nude mouse (B-16).

Saturation Density. Table 3 ranks the various cell lines in order of decreasing saturation density and indicates their tumorigenicity in athymic nude mice. The data show no apparent density threshold for tumorigenicity. The saturation density of 3T6 cells derived from a nude mouse tumor (3T6T) is not greater than that of the parental 3T6 cells that generated the tumor.

Anchorage Requirement. Table 4 ranks the cells lines in order of increasing anchorage requirement. No association between anchorage requirement and tumorigenicity is revealed by the data. The 3T6 line grew extremely well in nude mice, an inoculum of 10^6 cells forming a visible tumor within 5 days, yet 3T6 cells did not grow at all in Methocel. An inoculum of 10^5 cells yielded no colonies and, by phase-contrast microscopy, it could be seen that individual cells did not divide even once after 3 weeks in culture. 3T6 cells recovered from a nude mouse tumor (3T6T) showed the

Table 2
Relationship of serum growth requirement to tumorigenicity in nude mice

Cell line	Population-doubling time (hr)		Ratio of doubling times (10% FBS/1% FBS)	Tumorigenic in nude mice
	10% FBS	1% FBS		
HeLa	27.8	27.8	1.0	Yes
BRLC	45.6	48.0	0.95	No
3T6	14.4	18.2	0.79	Yes
PY-3T3	29.8	39.3	0.75	Yes
BRL-4143 Hiene	81.6	113.0	0.72	Yes
B-16T	15.6	27.2	0.57	Yes
SVT2	17.0	30.2	0.56	Yes
A-9	15.9	30.7	0.52	Yes
MDCK	18.0	38.4	0.47	No
B-16	21.6	54.2	0.40	Yes
C-6	22.3	54.2	0.40	Yes
HFL-Johnson	30.7	143.0	0.21	No
3T3	32.6	∞	0.00	No
VA2-8-aza-G^r	48.0	∞	0.00	No
LNSV	47.5	∞	0.00	No
RPMI-2650	26.5	∞	0.00	Yes
Embryonic mouse	50.4	∞	0.00	No
31A	24.0	∞	0.00	No

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same strong anchorage requirement as did the parental 3T6 line.

Contact Inhibition. The plating efficiencies of various cell lines on nongrowing mouse monolayers are summarized in Table 5. Line 31A had a high cloning efficiency on mouse monolayers but was not tumorigenic. Four cell lines and 1 cell strain (HFL-Johnson) did not form visible colonies; 3 of these same cell lines also would not form tumors in nude mice. These latter 3 lines (MDCK, VA2-8-aza-G^r, and 3T3) were assayed for growth by monitoring colony-forming units as described in "Materials and Methods" and the data are presented in Table 6. Only 1 cell line, 3T3, was unable to proliferate on top of irradiated mouse monolayer cultures. The VA2-8-aza-G^r and MDCK lines showed significant growth on top of mouse monolayer cultures, although macroscopically visible colonies did not form. The data thus show that the ability of cells to form colonies or to proliferate on top of mouse monolayers is not a sufficient condition for predicting tumor formation in nude mice.

Table 3

Relationship between saturation density and tumorigenicity in nude mice

Cell line	Saturation density, 10% FBS (cells/sq cm)	Tumorigenic in nude mice
RPMI-2650	1.5×10^6	Yes
A-9	8.5×10^5	Yes
VA2-8-aza-G^r	7.4×10^5	No
31A	7.3×10^5	No
C-6	6.1×10^5	Yes
HeLa	5.6×10^5	Yes
SVT2	3.7×10^5	Yes
MDCK	3.3×10^5	No
3T6	2.9×10^5	Yes
B-16	2.8×10^5	Yes
LNSV	2.4×10^5	No
PY-3T3	2.1×10^5	Yes
BRLC	2.0×10^5	No
3T6-tumor	1.8×10^5	Yes
HFL-Johnson	1.5×10^5	No
Embryonic mouse	7.0×10^4	No
Hiene	6.6×10^4	Yes
3T3	3.9×10^4	No

DISCUSSION

In a previous report (18), a variety of heterologous cell lines were categorized as tumorigenic or nontumorigenic in athymic nude mice. Evidence was presented to show that growth in the nude mouse is a reliable indicator of the malignant capability of a cell. The experiments described in this report show that, except for the 3T3 line and embryonic

Table 4

Relationship between anchorage requirement and tumorigenicity in nude mice

Cell line	% cloning efficiency: Methocel	% cloning efficiency: Plastic	Cloning efficiency (Methocel ÷ plastic)	Tumorigenic in nude mice
HeLa	12	7.6	1.6	Yes
A-9	30	46	0.65	Yes
C-6	31	60	0.52	Yes
MDCK	2.7	11	0.25	No
VA2-8-aza-G^r	1.8	10	0.18	No
PY-3T3	2.1	12	0.17	Yes
RPMI-2650	1.7	10	0.16	Yes
B-16	0.3	9.4	0.06	Yes
RB-SV$_3$	0.03	0.76	0.04	No
SVT2	0.01	10	0.001	Yes
3T3	<0.001	10	<0.0001	No
31A	<0.001	44	<0.0001	No
BRLC	<0.001	16	<0.0001	No
3T6	<0.001	25	<0.0001	Yes
3T6T	<0.001	12.6	<0.0001	

Table 5

Relationship between contact inhibition of growth and tumorigenicity in nude mice

Cell line	% cloning efficiency: Mouse monolayer	% cloning efficiency: Plastic	Cloning efficiency (monolayer ÷ plastic)	Tumorigenic in nude mice
A-9	40	40	1.9	Yes
31A	42	59	1.4	No
RPMI-2650	15	18	1.2	Yes
B-16	11	11	1.0	Yes
SVT2	31	34	0.91	Yes
C-6	21	40	0.52	Yes
MDCK	NVC[a]	11	0.0	No
PY-3T3	NVC	5.6	0.0	Yes
HFL-Johnson	NVC	3.4	0.0	No
VA2-8-aza-G^r	NVC	10	0.0	No
3T3	NVC	10	0.0	No

[a] NVC, no visible colonies were formed.

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Table 6

Cell proliferation on monolayer cultures of irradiated mouse embryo fibroblasts

Three nontumorigenic cell lines that did not form visible colonies on top of irradiated mouse embryo fibroblasts (see Table 5) were assayed for proliferation as described in "Materials and Methods." Proliferation is expressed as the fold increase in initial cell inoculum over a 4-day incubation period with 1 medium change at Day 2.

	Fold increase in clone-forming cells/culture			
Cell line	Irradiated fibroblasts	Bare culture dish	Ratio of fibroblasts/bare dish	Tumorigenic in nude mice
MDCK	5.9	48	0.12	No
VA2-8-aza-G^r	3.4	3.0	1.1	No
3T3	0.87	22	0.04	No

fibroblast strains, all of the cell lines that do not form tumors in nude mice nonetheless display 1 or more of the growth characteristics *in vitro* that have been associated with the transformed state. None of these growth parameters is consistently indicative of tumorigenic potential. These findings should underscore warnings by other investigators (2, 14) that the *in vitro* growth criteria commonly used to define the transformed state may not be related to growth regulation and oncogenesis *in vivo*.

A consistent goal of cancer biologists has been to discover a cellular function that would qualitatively distinguish all cancer cells from all normal cells. If one assumes that such a cancer-specific function exists, there are several obstacles to discerning its nature through comparative studies of cells in culture.

First, the behavior of cells in culture is very much affected by culture conditions. Saturation density may be affected by serum concentration (9) and by pH (3). Anchorage requirement may be determined by the capacity of different sera to permit high levels of plasmin formation (12). Serum requirement is partially dependent on the serum source; mouse serum, for instance, is more potent than bovine serum for supporting growth of 3T3 cells (9). Furthermore, as observed in these studies and in others (7, 11), the serum requirement of cells is a sensitive function of cell population density in culture.

Second, a particular phenotype in cell culture may be achieved by more than 1 mechanism. Vogel and Pollack (24) have shown 3 distinct mechanisms whereby cell cultures can maintain a constant cell population under nonpermissive growth conditions. Growth in low serum may indicate that a cell line has partially lost its requirement for growth-promoting serum hormones or, alternatively, that the cells are capable of synthesizing these factors for themselves. The very low serum requirement of the BRLC line, for example, probably reflects the fact that these cells synthesize and secrete somatomedins (6).

A 3rd consideration is that, while the spectrum of tumor-derived or oncogen-transformed cells available for study is wide, the nature and number of cell lines considered "normal" are rather narrow. Comparative growth and tumorigenicity studies have generally been conducted with primary or secondary embryo cells, 3T3 cells, or BHK cells as the normal reference. As generalities are established with these systems, it will be important to determine whether they extend to cells of epithelial origin. In this study and a previous report (18), we have examined the growth behavior and tumorigenicity of epithelioid cell lines derived from nonmalignant tissue, namely, 31A, MDCK, and BRLC. Although none of these lines was tumorigenic, all displayed 1 or more of the growth characteristics associated with the transformed state suggesting that embryo cells and fibroblast-like cell lines may be responsive to a rather specialized set of growth regulatory signals.

We conclude, in agreement with Sanford (14), that the term "transformed" should be restricted to those cells demonstrated to grow as neoplasms *in vivo* or be qualified to indicate the type of change observed. We further suggest, on the basis of this work and previous studies (18, 19), that tumorigenicity in congenitally athymic nude mice is presently the most reliable and physiologically meaningful assay for transformation.

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