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Correlation of patterns of anchorage-independent growth with *in vivo* behavior of cells from a murine fibrosarcoma

(metastatic potential/*in vivo* growth characteristics)

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ABSTRACT **The pattern of *in vitro* anchorage-independent growth of tumor cells from the murine UV-2237 fibrosarcoma correlated with their ability to produce experimental metastasis *in vivo*. When seeded into 0.3% Noble agar semisolid medium, cells of metastatic clones developed into larger tumor colonies at a faster rate than did cells of clones with low metastatic potential. Furthermore, when tumor cells were plated into 0.6% Noble agar, colony development by cells of low metastatic potential clones was almost completely restricted. Tumor cells from the heterogeneous parent UV-2237 fibrosarcoma were plated into dishes containing 0.6% agar semisolid medium. In separate experiments, 16 colonies were isolated 2 weeks thereafter and were established as individual cell lines in monolayer cultures. All of these cell lines produced experimental metastases as determined by *in vivo* lung colony assay. The data suggest that anchorage-independent growth of UV-2237 tumor cells in 0.6% Noble agar semisolid medium is selective and permits the isolation of metastatic subpopulations.**

Clinical and morphological observations of neoplasms have suggested that tumors may progress from benign (noninvasive, low metastatic potential) to malignant (invasive, metastatic) over a period of time (1–4). Tumor progression was originally defined by Foulds (1, 2) as the gradual evolution of a tumor toward increased autonomy by a series of stepwise changes in independent unit characteristics. The progression of tumors, therefore, is attributed to the emergence of new variant cells that have selective advantage for growth in their host (3, 5). It has been reported recently that several murine tumors are heterogeneous and contain subpopulations of cells with different metastatic potentials (6–10). This conclusion was based on the results obtained from *in vivo* studies in which a large number of syngeneic recipient animals injected subcutaneously (s.c.) or intravenously (i.v.) with different cell populations (isolated from a single tumor) were monitored for tumor metastases. Such *in vivo* studies have numerous limitations (11) and exclude altogether those investigations using tumors for which no syngeneic recipients are available. Therefore, it would be desirable to develop *in vitro* methods to isolate metastatic cells and then to identify *in vitro* properties that distinguish them from their tumorigenic counterparts with low metastatic potential.

One *in vitro* property of tumorigenic cells is their ability to grow progressively while suspended in semisolid medium (anchorage-independent growth). This property generally distinguishes transformed (tumorigenic) from normal (nontumorigenic) cells (12–17). This characteristic of tumorigenic cells as well as their lack of contact inhibition of growth and low serum requirements have been attributed to an increase in autonomy from growth regulatory mechanisms. It has been proposed that multiple cellular changes are required for anchorage-independent growth to occur (17). Because the evolution of tumors is thought to be gradual and proceeds toward increasing autonomy (3), we questioned whether the pattern of *in vitro* anchorage-independent growth can distinguish between tumorigenic cells that are metastatic and those that are of low metastatic potential. Recent cloning studies demonstrated that a fibrosarcoma induced in a C3H mouse by chronic irradiation with UV light is heterogeneous and contains subpopulations of both metastatic and low metastatic potential (6). The availability of these clones, which breed true upon recloning, allowed us to determine whether the pattern of *in vitro* anchorage-independent growth of tumor cells seeded into semisolid medium (0.3% Noble agar) correlated in some way with their metastatic potential. We also investigated whether the ability of tumor cells to multiply and to develop into colonies in a more restricted semisolid medium (containing 0.6% Noble agar) could be used as a method for the isolation of metastatic tumor cells from the parental heterogeneous UV-2237 fibrosarcoma.

MATERIALS AND METHODS

Cell Cultures. The UV-2237 is a fibrosarcoma that was induced by chronic UV irradiation of a female C3H$^-$ mouse. The tumor was established in culture from the first *in vivo* passage in immunosuppressed syngeneic mice, and cloned cell lines from the sixth *in vitro* passage were produced as described (6). All cell lines were grown as monolayers on plastic flasks in Autopow minimal essential medium (Flow Laboratories, McLean, VA) supplemented with 10% fetal calf serum, glutamine, nonessential amino acids, and vitamins (GIBCO) and designated "complete minimal essential medium." The cultures were maintained at 37°C in a humidified incubator in an atmosphere containing 5% CO_2. All cell lines were examined and found free of *Mycoplasma* and the following murine viruses: reovirus type 3, pneumonia virus of mice, K virus, Theiler's virus, Sendai virus, minute virus of mice, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (Microbiological Associates, Walkersville, MD). In order to ensure reproducibility of *in vivo* and *in vitro* assays, the cultures were tested within 4 weeks after recovery from frozen stocks.

For *in vivo* (experimental metastasis) and *in vitro* studies, tumor cells were always harvested from subconfluent cultures (50–70% confluence) by rinsing the monolayers with a 0.25% trypsin/0.02% EDTA. After 1 min, the flasks were tapped sharply to dislodge the monolayers, and the cells were pipetted gently into the complete medium containing 10% fetal calf serum. Cells used for i.v. injection were resuspended in Hanks'

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Abbreviations: i.v., intravenous(ly); s.c., subcutaneous(ly).

Table 1. Size of colonies and colony formation efficiency of UV-2237 fibrosarcoma cells with different metastatic potentials, at 14 days after plating

Clone	Metastatic potential	Cloning efficiency*, %		Doubling time in monolayer, hr†	Distribution of colonies grown in agar, by diameter at 2 weeks, %‡			
		In monolayer	In agar		0.05–0.15 mm	0.16–0.30 mm	0.31–0.50 mm	>0.50 mm
15	Low	35	9	20.3	97	2	1	0
38	Low	5	1	17.4	100	0	0	0
42	Low	39	14	—	89	9	2	0
43	Intermediate	23	9	18.7	78	11	11	4
39	High	31	11	21.7	78	3	3	13
25	High	25	13	24.4	37	16	16	17

* Cells at two different concentrations (375 and 675) were plated into 60-mm culture dishes in monolayer and in 0.3% agar. The number of monolayer colonies (>25 cells each) was determined 8 days later. The number of colonies growing in agar >0.05 mm in diameter was determined 21 days after plating.

† Calculated from the slope of a 96-hr growth curve of cells in logarithmic growth.

‡ Single cells (1500) were plated in 0.3% Noble agar in 60-mm culture dishes in triplicate. The diameters of at least 100 colonies were measured. Underlayers were 0.6% Noble agar.

balanced salt solution (11). Only suspensions containing single cells of >90% viability were used in the experiments.

Clones of the UV-2237 fibrosarcoma were classified as having low, intermediate, or high metastatic potential based on their behavior in three different *in vivo* tests. The first test was the quantitative lung colony assay in which mice were given i.v. injections of tumor cells, and the number of lung colonies was counted 3 weeks later. In the second test, mice were given s.c. injections of tumor cells, and the number and site of spontaneous metastases were determined at autopsy. In the third test, mice were given i.v. injections of tumor cells, and the number and site of extrapulmonary tumors were determined (6). The three tests gave similar results in that clones judged to be of high or low metastatic potential in one test usually exhibited the same behavior in the other two tests. The clones were ranked in order of increasing metastatic potential. Summing the rank of each clone in each of the three tests gave a relative measure of how metastatic each clone was, based on all three tests (6).

Determination of Growth Rate in Monolayer Cultures. Cell lines were plated at a density of 10^4 cells per 60-mm plastic petri dish (Falcon). Duplicate cultures were trypsinized, and the number of cells per dish was determined every 24 hr for 5 days by using a Coulter Counter.

Growth in Semisolid Medium. A modification of the technique of MacPherson was used (12). Base layers (4 ml) of complete Autopow minimal essential medium with 10% tryptose phosphate broth containing 0.6% Difco Noble agar were set in 60-mm plastic dishes. This was overlaid with 1.5 ml of a second layer of agar containing a suspension of 1500 single cells (375 or 675 cells for experiments measuring plating efficiencies). The concentration of the top agar layer varied among experiments and ranged from 0.2 to 0.6%. The diameter of tumor colonies was determined with a microscope equipped with a Filar micrometer eyepiece (American Optical). Because not all colonies were spherical, the longest diameter of each colony was measured. All cultures were done in triplicate and at least 100 colonies per group were measured at each time point.

In several experiments, the UV-2237 parent tumor cells were seeded into semisolid 0.6% agar. Fourteen days later, those colonies with a diameter exceeding 0.4 mm were removed and placed into 16-mm plastic culture dishes. Two to 3 weeks thereafter, the lung colonization capacity of the cells growing in monolayer cultures was tested as described below.

Animals. Specific-pathogen-free C3H/HeN (MTV^-) ($C3H^-$) mice were obtained from the Animal Production Area of the Frederick Cancer Research Center. Mice were irradiated with UV light for 1 hr three times per week for 8 weeks, as detailed (18). None of the animals had developed primary tumors from the UV irradiation at the time of the experiments. Age- and sex-matched untreated mice served as controls for the UV-treated groups.

Experimental Pulmonary Metastasis. Unanesthetized normal or UV-irradiated mice were inoculated i.v. with 1×10^5 tumor cells in 0.2 ml of Hanks' balanced salt solution via the tail vein. All mice were killed 21 days after tumor cell injection, and their lungs were removed, rinsed in water, and fixed overnight in Bouin's solution. This procedure renders the uninvolved lung parenchyma yellow, providing sufficient contrast to the white tumor colonies for visual counting (11). The number of tumor colonies was determined by counting surface metastases under a dissecting microscope; the majority of such experimental metastases in mice are found on the surface of the lungs (11). The lung colonies were counted in a blind fashion by two observers.

RESULTS

Growth of UV-2237 Fibrosarcoma Clones in Noble Agar. Single cells from three clones of low metastatic potential, one clone of intermediate metastatic potential, and two highly metastatic clones were grown suspended in 0.3% Noble agar medium over a 0.6% Noble agar underlayer. The diameters of

Table 2. Relationship of culture time to size of tumor colonies growing in 0.3% agar

Clone	Culture day	Metastatic potential	Distribution by colony size, %			
			0.05–0.15 mm	0.16–0.3 mm	0.31–0.50 mm	>0.50 mm
15	9	Low	99	1	0	0
	14		97	2	1	0
	21		86	6	4	4
43	9	Intermediate	88	10	1	1
	14		79	11	7	4
	21		62	20	8	10
39	9	High	90	2	4	4
	14		83	6	4	7
	21		72	10	6	12

Single cells (1500) were plated in triplicate in 60-mm culture dishes.

Table 3. Effect of agar concentration on the development and size of tumor colonies

Clone	Metastatic potential	Agar conc. in top layer, %	Distribution by colony size, % 0.05–0.15 mm	0.16–0.30 mm	0.31–0.50 mm	>0.50 mm
15	Low	0.2	84	13	3	0
		0.3	97	3	0	0
		0.4	100	0	0	0
		0.5	100	0	0	0
		0.6	Single cells	0	0	0
39	High	0.2	—	—	—	—
		0.3	56	26	13	5
		0.4	83	8	6	4
		0.5	88	5	1	5
		0.6	89	3	2	6
43	Intermediate	0.2	46	23	15	15
		0.3	55	23	10	12
		0.4	61	20	9	9
		0.5	65	16	9	9
		0.6	76	9	9	6

Single cells (1500) were plated in the indicated agar concentration in 60-mm culture dishes in triplicate. All underlayer agar concentrations were kept at 0.6% Noble agar. Fourteen days after plating, at least 100 colonies were measured.

the resulting colonies were measured 14 days after plating. The size distribution of colonies varied within and among the clones. A representative experiment is shown in Table 1. Tumor colonies ranging in diameter from 0.05 to 0.3 mm were observed in agar dishes seeded with cells from low, intermediate, and high metastatic potential clones. In contrast, tumor colonies >0.31 mm in diameter were observed more frequently in dishes with cells from metastatic clones.

The differences in colony size among the clones could not be attributed to preferential cell clumping of metastatic cells, cloning efficiency, or cell doubling time. There were no significant differences among the clones (within a confidence limit of 95%) in doubling time measured during logarithmic growth in monolayer cultures (Table 1).

The diameter of tumor colonies growing in agar increased with the length of incubation. By day 14 after plating, colonies >0.3 mm in diameter were observed more frequently with clones 43 and 39 than with clone 15 (Table 2). By day 21 after plating, however, large tumor colonies were found with cells of all three clones, suggesting that the differences in colony size are due to differences in the time required for the appearance of large colonies rather than to the inability of cells from clone 15 to develop into colonies >0.3 mm in diameter.

In the preceding experiments, tumor cells were suspended in a 0.3% Noble agar medium and plated on top of a 0.6% Noble agar underlayer. In order to determine the optimal conditions for colony growth, we varied the concentrations of the agar used in the assay. Varying the concentration of the agar in the bottom layer had little effect on cloning efficiency or colony size distribution of the clones tested. We therefore continued to use 0.6% agar as an underlayer. In contrast, varying the agar concentration of the top layer of agar medium (into which the cells were suspended) had a marked effect. An increase in agar concentration limited the development of visible colonies (>0.3

Table 4. Size of tumor colonies at 14 days after plating of cells from parent UV-2237 fibrosarcoma and cloned subpopulations into 0.6% Noble agar

Cell source	Metastatic potential	Distribution by diameter of agar colonies, % 0.05–0.15 mm	0.16–0.30 mm	0.31–0.50 mm	>0.50 mm
Parent UV-2237	High	72	11	10	7
Clone 15	Low	100	0	0	0
Clone 38	Low	100	0	0	0
Clone 42	Low	98	1	1	0
Clone 22	Intermediate	85	13	2	0
Clone 41	Intermediate	72	15	4	9
Clone 12	Intermediate	86	8	1	5
Clone 43	Intermediate	76	16	5	3
Clone 39	High	91	6	1	2
Clone 34	High	61	24	13	2
Clone 25	High	90	6	2	2
Clone 31	High	59	16	13	12

Cells (1500 per 60-mm dish) were plated in 1.5 ml of 0.6% Noble agar on a 4-ml underlayer of 0.6% agar. Plates were incubated in a humidified 37°C incubator. At 14 days the visible colonies were noted and 200 colonies were measured by using a micrometer eyepiece.

mm) to dishes containing cells of intermediate or high metastatic potential (Table 3).

The diameter of tumor colonies in all groups (measured 14 days after plating) decreased with increasing agar concentrations. An interesting effect, however, was observed in dishes seeded with cells of clone 15 (low metastasic potential). When those cells were suspended in medium containing agar concentrations >0.4%, no colonies >0.15 mm in diameter were detected. In contrast, visible tumor colonies (>0.3 mm) were observed in dishes seeded with cells of clones 43 and 39 in 0.6% agar medium (Table 3).

UV-2237 Fibrosarcoma Clones in Restrictive 0.6% Noble Agar. Cells from the parent UV-2237 fibrosarcoma and eight cloned cell lines were plated into 0.6% Noble agar medium. The diameters of 200 colonies per test group were measured 2 weeks after plating. There was a correlation between the ability of tumor cells to develop into visible tumor colonies (>0.3 mm) in 0.6% Noble agar and the formation of visible pulmonary metastases after i.v. injection (Table 4). The presence of visible colonies at day 14 in 0.6% Noble agar medium separates the clones into two major categories: those that frequently metastasize *in vivo* and those that rarely metastasize. These results were unaltered even when cultures were terminated on day 21. Low-metastatic-potential lines still did not form visible colonies (data not shown).

Growth of Parental UV-2237 in 0.6% Agar. We wished to determine whether the ability of cells to grow rapidly in a 0.6% agar medium could predict their behavior *in vivo* after i.v. injection. Cells from the heterogeneous parent UV-2237 tumor were seeded into 0.6% agar medium. Fourteen days later, eight colonies >0.5 mm in diameter (Exp. 1) and another eight colonies >0.4 mm in diameter (Exp. 2) were isolated and grown in monolayer cultures to expand their number. The individual monolayers were harvested, and 10^5 single viable tumor cells were injected i.v. into groups of 10 normal (Exps. 1 and 2) and 5 UV-irradiated C3H$^-$ mice (Exp. 1). [Because UV-induced tumors are known to be immunogenic, the UV-treated (immunosuppressed) mice were included as controls.] Cells of the parent UV-2237 tumor and clones 15 and 38 (low metastatic potential) served as additional controls. The mice were killed 21 days after injection and the number of pulmonary metastases was determined as described above.

Cells obtained from all eight colonies in Exp. 1 and all eight colonies in Exp. 2 yielded pulmonary metastases in normal and UV-irradiated mice; control cells of the low-metastatic-potential clones 15 and 38 did not (Table 5). Here again, the growth of tumor cells into colonies in 0.6% agar medium did not distinguish between cells with high or intermediate metastatic potential but rather correlated with their ability to form metastases after i.v. injection.

Table 5. Experimental pulmonary metastasis produced by UV-2237 fibrosarcoma cells isolated on day 14 from colonies growing in 0.6% Noble agar

Cell source	Median (range) number of pulmonary metastases in: Normal mice	UV-irradiated mice
	Experiment 1	
Parent UV-2237	25 (18–48)	28 (16–39)
Colony 9	4 (2–6)	4 (2–7)
2	6 (4–8)	10 (3–20)
6	6 (3–38)	12 (7–41)
1	7 (6–10)	9 (3–13)
5	10 (6–24)	17 (6–37)
8	19 (4–30)	23 (11–33)
4	21 (16–61)	35 (14–42)
3	23 (2–31)	43 (17–55)
Clone 15 (low)	1 (0–4)	1 (0–6)
Clone 38 (low)	0 (0–1)	0 (0–1)
	Experiment 2	
Parent UV-2237	57 (5–145)	
Colony 14	10 (3–23)	
18	12 (2–24)	
17	18 (3–53)	
11	23 (4–80)	
13	24 (5–47)	
16	25 (7–61)	
15	104 (60–170)	
12	145 (50–210)	
Clone 15 (low)	0.5 (0–7)	
Clone 25 (high)	67 (14–166)	

Viable tumor cells (1×10^5 single cells) were injected i.v. into syngeneic recipients (n = 10 for normal and 5 for UV-irradiated mice). All mice were killed 21 days after injection and the number of pulmonary metastases was determined with a dissecting microscope.

DISCUSSION

Our results indicate that the pattern of anchorage-independent growth of cells from clones of the murine fibrosarcoma UV-2237 distinguishes cells that frequently metastasize from those that do not. Moreover, when single cells from this heterogeneous tumor (6) are seeded into semisolid medium containing 0.6% Noble agar, the growth of low-metastatic-potential cells is almost completely restricted and, therefore, metastatic subpopulations can be identified (Table 5).

In our studies, all tumorigenic cells—i.e., low metastatic potential or metastatic—could grow in the standard 0.3% Noble agar semisolid medium. By 14 days after plating, numerous tumor colonies with diameters >0.3 mm were observed in plates seeded with cells from metastatic clones. In general, during the same time period, cells of low-metastatic-potential clones did not develop into tumor colonies of comparable size. When the agar concentration was increased to 0.6%, cells of metastatic clones could be distinguished from cells of low metastatic potential by the ability of the former to multiply and develop into visible colonies. Only rarely did a colony >0.3 mm occur in dishes seeded with cells of a low-metastatic-potential clone. Occasionally, a tumor colony developed in a dish seeded with cells from a line of low metastatic potential (clone 42, Table 4). This correlates with the infrequent metastases produced *in vivo* by such a clone (6). Nonetheless, the growth in 0.6% agar is selective and limits growth of cells from clones that do not produce frequent metastases. The extent of this growth limitation can vary (clone 15, Tables 3 and 4) but is always pronounced when compared to colony development of metastatic cells.

The basis of the differences in anchorage-independent growth that we observed among clones with different metastatic potential *in vivo* is unclear. The doubling time of cells from metastatic or low-metastatic-potential clones growing in monolayer cultures is very similar (Table 1) (19), but whether it can be compared to the doubling time of cells growing in soft agar is unknown. The rate of tumor colony development also depends on the size of the growth fraction of the colony, which is the proportion of cells undergoing division at a given time. The growth fraction of tumor spheroids (colonies) developing from single cells has been shown to differ among several murine tumors (20, 21), which could be due to the ability of cells to

respond to exogenous or endogenous growth-regulating factors (22). It is also possible that the differences in colony size distributions among the clones may be unrelated to growth rate *per se* but may reflect differences in lag periods before the onset of cell division.

Neoplastic lesions produced by the i.v. injection of UV-2237 cells are referred to as metastases. The introduction of cells into the circulation bypasses the need for initial detachment of cells from the primary tumor and invasion of blood vessels. Although all subsequent steps in the metastatic process must occur for i.v. injected cells to establish a metastasis, elimination of these initial steps introduces the risk that noninvasive tumor cells might form metastases when injected i.v. but be unable to metastasize spontaneously when implanted s.c. or intramuscularly. We have recently addressed this question by comparing the metastatic behavior of the UV-2237 clones after i.v. or s.c. inoculation. A strong positive correlation was observed for the ranking of the clones in increasing order of metastatic behavior. Clones that were judged to be of low metastatic potential by one test were also of low metastatic potential by the other tests (6). This indicates that the formation of experimental metastases by i.v.-injected tumor cells is a valid assay of cellular metastatic potential and parallels results obtained in the more tedious and time-consuming assays of spontaneous metastasis formation.

Quantitative studies of metastasis indicate that a tumor focus in a distant organ can result from the survival of even a single cell (7, 11). Therefore, the final step in the metastatic process—i.e., multiplication and proliferation of cells—is crucial to the development of a visible lesion. We speculate that the ability of single cells from the UV-2237 fibrosarcoma to grow in a restrictive (0.6%) Noble agar medium may correlate with their ability to multiply *in vivo* from single cells or clumps of cells into metastases in the parenchyma of organs such as the lungs. The proliferative capability of cells does not ensure development of a metastasis. Because the pathogenesis of metastasis depends on the successful completion of a multistep process, growth in 0.6% Noble agar might not correlate with all such steps. For example, cells that are capable of proliferation *per se* but cannot survive transport in the circulation or cannot invade surrounding tissues or cells that are vulnerable to destruction by the host immune system might form colonies in 0.6% agar but would not yield metastases in an *in vivo* assay.

Murine tumors induced by chronic UV irradiation can be immunologically rejected upon transplantation to normal syngeneic mice (18, 23). These tumors will grow progressively in syngeneic mice that have been treated for a short period with UV irradiation (23). We tested the metastatic potential of cells from colonies isolated in 0.6% Noble agar in both normal and UV-irradiated recipients. As shown in Table 5, cells of all 16 colonies produced lung metastases after i.v. injection into animals of either test group. In contrast, 6 of 21 clones of the UV-2237 produced on acrylamide grids were shown to be of low metastatic potential (6).

We conclude that the pattern of *in vitro* anchorage-independent growth of cells from clones of UV-2237 fibrosarcoma correlates with their *in vivo* behavior. Furthermore, the growth of tumorigenic cells in 0.6% Noble agar medium provides a method for the isolation (cloning) of metastatic tumor cells from the parental heterogeneous UV-2237 tumor. The *in vitro* studies described here should be tested in various other tumor systems in order to test the generality of our findings.

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