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Increasing metastatic potential is associated with increasing genetic instability of clones isolated from murine neoplasms

(cancer metastasis/progression/rate of mutation)

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ABSTRACT **The metastatic stability of clones, which were derived from the murine UV-2237 fibrosarcoma and which exhibit low or high metastatic potential, was examined after 60–72 days of continuous growth *in vitro* and *in vivo*. Subclones of the high metastatic clone exhibited a 140-fold variation in the production of experimental pulmonary metastases after intravenous injection into syngeneic C3H$^-$ mice. In contrast, subclones from the low metastatic clone varied only slightly (8-fold). Using cloned cells from three mouse tumors with differing metastatic potential, we determined the spontaneous mutation rates of cells with low or high metastatic capacities with respect to the selective genetic markers, 6-thiopurine resistance or ouabain resistance, or both. In all cases, cells with high metastatic potential had a 3- to 7-fold increase in the rate of mutation (per cell generation) at both genetic loci, as compared with their low metastatic but tumorigenic cell controls. These results support the hypothesis that the evolution of tumors from the benign to the malignant state could be the consequence of acquired genetic instability in the neoplastic cells.**

At the time of diagnosis, many human and animal tumors are heterogeneous and contain numerous subpopulations of cells with different biological characteristics, including metastatic potential (1–3). In some tumors, this biological diversity results from their multicellular origin (4) but, in other tumors that originate from a single transformed cell (5, 6), the source of the biological diversity is less clear. The evolution of tumors or their progression (7, 8) from the benign to the malignant state has been attributed by Nowell (9) to be the consequence of acquired genetic variation in the cells populating a neoplasm. Tumor cells are thought to be less stable genetically than normal cells (10), and the spontaneous mutation rate of virally transformed cells can increase with each level of transformation (11). This increased genetic instability could produce new variants (clones, sublines) within the developing neoplasm. These clones would be subjected to environmental selection pressures (12, 13) that favor the survival of variants with increased malignant capacity.

Nowell's hypothesis for tumor progression (9) predicts that those tumor cells that progress to an advanced stage of malignancy (i.e., metastatic cells) would be less stable genetically than nonmetastatic tumor cells. In this study, we have examined Nowell's hypothesis by determining the phenotypic stability of metastatic and nonmetastatic tumor cell lines by counting the number of pulmonary metastases that they produce after intravenous injection. In addition, we have compared the rates of mutation to ouabain resistance or 6-thiopurine resistance, or both, of high metastatic (HM) cells from three different murine tumors to that of low metastatic (LM) or nonmetastatic tumorigenic cells isolated from the same neoplasms.

MATERIALS AND METHODS

Animals. Six- to 8-week-old specific pathogen-free C3H/HeN mammary tumor virus negative (C3H$^-$) female mice were obtained from the Animal Production Area of the Frederick Cancer Research Center. In individual experiments, all mice were matched by age.

Cell Cultures. The UV-2237 is a fibrosarcoma that was induced in a female C3H$^-$ mouse by chronic UV irradiation (14). The tumor was established in culture from the first *in vivo* passage in immunosuppressed syngeneic mice. Cloned cell lines were obtained from the sixth *in vitro* passage as described (14). The K-1735 melanoma developed in a C3H$^-$ female mouse that had been treated with a short course of exposures to UV radiation, followed by chronic painting of the skin with croton oil (15). The primary tumor was adapted to growth in culture and cloned as described (16). SF-19 is a spontaneous fibrosarcoma that arose in a female C3H$^-$ mouse and was established in culture (17). A line of SF-19 tumor cells with HM propensity was derived from the parent SF-19 tumor after its exposure *in vitro* to nine cycles of UV radiation (18). All cell lines were grown as monolayers on tissue culture plastic dishes in McCoy's 5A medium (Flow Laboratories, McLean, VA) supplemented with 10% (vol/vol) fetal bovine serum, glutamine, and gentamicin sulfate (Shering, Kenilworth, NJ). The cultures were maintained at 37°C in a humidified incubator in 95% air/5% CO_2. All cell lines were tested and found to be 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 (MA Bioproducts, Walkersville, MD). In order to ensure the reproducibility of the *in vitro* assays, the cultures were used within 4 wk 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 0.25% trypsin/0.02% EDTA. After 1 min, the flasks were tapped sharply to dislodge the monolayers, and the cells were pipetted carefully into medium containing 10% fetal bovine serum. Cells used for intravenous injections were resuspended in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution. Only suspensions containing single cells of >90% viability (as measured by trypan blue exclusion) were used in the experiments.

Experimental Pulmonary Metastasis. Unanesthetized mice were inoculated intravenously with 1×10^5 tumor cells suspended in 0.2 ml of Hanks' balanced salt solution through the lateral tail vein. All mice were killed 21 days after tumor cell

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Abbreviations: HM, high metastatic; LM, low metastatic.

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injection, and their lungs were removed, rinsed in water, and fixed overnight in Bouin's solution (19). Because most experimental metastases in mice are found on the surface of the lungs (19), the number of tumor colonies was determined by counting parietal metastases under a dissecting microscope. The number of lung metastases produced by individual clones and subclones was compared to that produced by parental populations using the Mann–Whitney U test to ascertain the significance of the differences observed (20).

Cloning Procedures. Cells were cloned by end-point dilution. Single cell suspensions of viable tumor cells were seeded into wells of a Falcon 3034 microtest plate at 0.5 cell per well. Wells containing single cells were marked, and the colonies were transferred after sufficient growth to vessels of increasing size to expand the cell population. The cloned cultures were frozen until needed for *in vivo* testing.

Fluctuation Analysis. For each clone or cell line studied, 2×10^3 cells were plated in 27 plastic Petri dishes (100 mm). After 7–10 days at 37°C in a humidified CO_2 incubator, two sample plates were fixed and stained with methylene blue dissolved in 50% (vol/vol) methanol, and colonies were counted to determine the initial cell number. Cells from the remaining 100-mm Petri dishes were trypsinized, transferred to 150-cm^2 Falcon flasks (one Petri dish per flask), and then incubated 4–5 days until there were approximately 10^7 cells per flask. The cells were trypsinized and counted; the total content of each flask was divided among 10 replicate 100-mm plastic Petri dishes containing medium and 5 mM ouabain (Sigma) or 4 μg of 6-thiopurine (Sigma) per ml. Parallel duplicate control cultures were counted and plated in medium without ouabain to determine the efficiency of colony formation. After 13–15 days at 37°C in a humidified CO_2 incubator, the cultures were fixed and stained with methylene blue in 50% methanol. The rate of mutation was calculated by the equation described by Luria and Delbrück in formula 8 of ref. 21: $r = aN_t\ln(N_tCa)$ where r is the average number of resistant cells per plate, a is the mutation rate (to be solved for), N_t is the number of cells in the growing cultures (at the time of testing), and C is the number of cultures (samples). Because in this equation a solves for the mutations per cell (bacterium) per time unit, we multiplied a by the natural logarithm of 2 (ln 2) to solve for the mutations per cell division cycle (cell generation), as explained by Luria and Delbrück (21). The formula used was:

$$r = \frac{a}{\ln 2} N_t \ln \left[(N_t)\,(C)\,\frac{(a)}{\ln 2} \right].$$

The numbers were adjusted for colony-forming efficiency.

RESULTS

The Stability of the Metastatic Phenotype in Cloned UV-2237 Cell Lines. We first assessed the stability of the metastatic phenotype in UV-2237 fibrosarcoma cloned cell lines with LM or HM potential. The clones were classified as having a LM or HM potential, based on their capacity to produce both spontaneous and experimental metastases (14). Cells of LM and HM clones were recovered from frozen stocks and subcloned within 1 wk after recovery. The LM and HM clones were cultured *in vitro* for 72 or 60 days, respectively. At these times, a series of subclones were isolated from both the clones and tested for their ability to produce experimental lung metastases. In parallel studies, cells from the LM or HM clone lines were injected subcutaneously into $C3H^-$ mice (10^4 cells per mouse). Developing tumors were excised surgically on day 72 (LM) or day 60 (HM). Cell cultures were established from the subcutaneous tumors by enzymatic dispersal of tumor tissue with 0.2% collagenase type I and 0.1% trypsin. One week later, a second series of subclones were isolated, and these also were tested for their ability to produce experimental lung metastases. Cells of the LM clone produced lung metastases at frequencies of one lung nodule per mouse per 10^5 cells injected (Fig. 1). In contrast, cells of the HM clone produced about 50 lung nodules per mouse per 10^5 cells injected. The data also show that the LM clone is relatively stable in its metastatic phenotype. Whether passaged *in vitro* (72 days, approximately 80 cell doublings) or grown *in vivo* (72 days), the majority of the subclones were indistinguishable from the parent LM clone for production of experimental metastases. Some of the subclones isolated from the *in vitro* or *in vivo* propagated lines showed a minor but significant shift ($P < 0.01$) toward a higher metastatic potential, but others exhibited a lower frequency of metastasis than the parent LM clone.

The results with the HM clone were strikingly different (Fig. 1). When the HM clone was thawed and subcloned immediately after becoming reestablished in cell culture, most subclones were indistinguishable from their parent in their ability to produce experimental metastases. However, after 60 days of growth *in vitro* (approximately 65 cell doublings) or 60 days of growth in the subcutaneous tissue, many subclones had metastatic properties that differed significantly from their parent clone. The median number of pulmonary metastases per mouse produced by cells of the different HM subclones varied as much as 140-fold (from 2 to 280 nodules per mouse), suggesting that the metastatic phenotype of the HM clone is unstable. Sixty days of growth of the HM clone *in vitro* or *in vivo* leads to the development of heterogeneity for metastasis that is similar to the heterogeneity originally found for the uncloned parent UV-

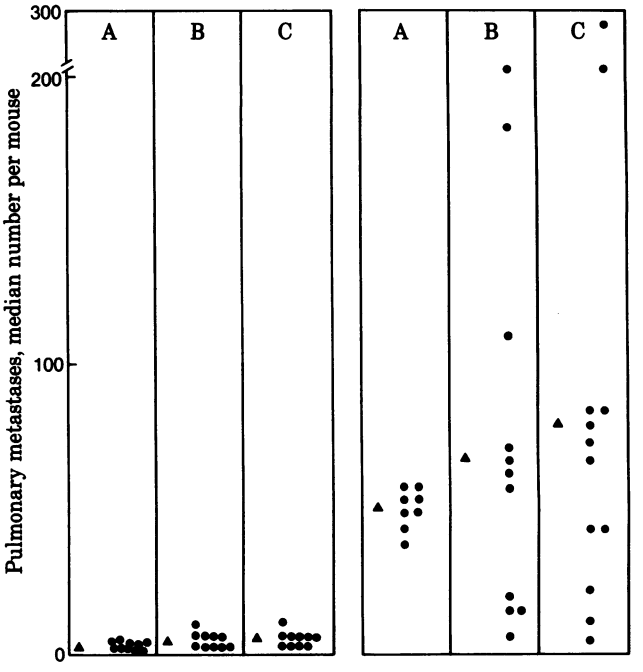

FIG. 1. Experimental metastasis produced by cells of individual subclones (●) isolated from parent UV-2237 clones (▲). Subcloning experiments were performed immediately after cells were recovered from frozen stocks (A) at 72 days (LM clone) (*Left*) or 60 days (HM clone) (*Right*) after the parent clones were grown in culture (B) or subcutaneously injected into syngeneic $C3H^-$ mice (C). The median number of pulmonary metastases per mouse was derived from the counts taken from at least 10 mice per group. Mice were injected intravenously with 10^5 cells and were killed 3 wk later; the experimental metastases were counted under a dissecting microscope (19).

Table 1. Rate of mutation to ouabain resistance or 6-thiopurine resistance of UV-2237 fibrosarcoma cells with LM or HM potential

Cell line	Metastatic potential*	Selective agent†	Rate of mutation‡	Fold increase, HM/LM
UV-2237-LM-1	Low	Ouabain	0.158	—
UV-2237-HM-2	High	Ouabain	0.728	4.6
UV-2237-LM	Low	6-Thiopurine	0.434	—
UV-2237-HM	High	6-Thiopurine	1.30	3.0

* See Fig. 1.
† Selection for mutants was performed in 5 mM ouabain or 4 μg of 6-thiopurine per ml.
‡ The rate of mutation (× 10^6 per cell generation) was calculated by the equation described by Luria and Delbrück (21).

Table 2. Rate of mutation to ouabain resistance of tumor cells with LM or HM potential

Cell line	Metastatic potential*	Rate of mutation†	Fold increase, HM/LM
UV-2237 LM-42	Low	0.0764	—
UV-2237 HM-39	High	0.502	6.5
K-1735 melanoma-LM	Low	0.0873	—
K-1735 melanoma-HM	High	0.610	7.0
SF-19	Low	0.0178	—
SF-19-UV9	High	0.103	5.8

* UV-2237 (14), K-1735 melanoma (16), SF-19 (24).
† The rate of mutation (× 10^6 per cell generation) was calculated as described (21).

2237 fibrosarcoma (14). The differences in the stability of metastatic properties between the LM and HM clones could not be attributed to differences in the number of cell doubling times. At least *in vitro*, the cell doubling times of these two lines are similar (20–24 hr) (22). Furthermore, the LM clone was cultured for a longer period before the subcloning experiments than was the HM clone.

Spontaneous Mutation Rates of Cells with Low and High Metastatic Potential. In an effort to determine the spontaneous mutation rates of cells from the UV-2237 LM and HM clone lines, we performed fluctuation analyses as described by Luria and Delbrück (21). Two well-characterized genetic markers were studied: resistance to the metabolite 6-thiopurine, involving a mutation in the gene for hypoxanthine/guanine phosphoribosyl-transferase, and resistance to the drug ouabain, involving an alteration in the cell membrane-bound Na^+,K^+-ATPase (23). Data from a representative experiment are shown in Table 1. The mutation rates for conversion both to 6-thiopurine resistance and ouabain resistance were higher for the HM cells than for the LM cells (3.0- and 4.6-fold, respectively). The differences in the rate of mutation to 6-thiopurine resistance could not be attributed to differences in the chromosome number of the clones. Both the LM and HM clones were primarily diploid. The mode and range of chromosome number for the LM clone was 39 (23–68) and for the HM clone was 40 (19–85) (22). The increased rate of mutation for metastatic cells relative to their nonmetastatic control was also found in three additional pairs of tumors. For these experiments, we analyzed a different set of UV-2237 fibrosarcoma clones with LM or HM potential (14), LM or HM clones of the K-1735 melanoma (16), and LM or HM cells of the SF-19 fibrosarcoma (18, 19) (Table 2). In all three systems, the rate of mutation to ouabain resistance of the metastatic cells was higher than that of their LM or nonmetastatic counterparts (6.5-, 7.0- and 5.8-fold, respectively). In all of the fluctuation assays we performed, some of which were repeated two or three times for a given set of cells, we never failed to detect at least a 3-fold greater rate of mutation (per cell generation) in metastatic cells than in their nonmetastatic cell controls. In each of the four pairs of LM or HM cells examined, the cell doubling times in monolayer cultures, as well as mean chromosome numbers, were very similar (data not shown). We also confirmed that the tumor colonies growing in the selected dishes were indeed resistant to the drugs ouabain or 6-thiopurine by isolating them and replating them in selective media.

DISCUSSION

Tumor progression has been attributed, in part, to a genetic mechanism (9). According to this hypothesis, progression occurs as a result of acquired genetic alterations in the developing tumor cells. It has been postulated that as a tumor progresses to an advanced state of malignancy, it contains cells that are more mutable than cells that did not progress as far and are still nonmetastatic (i.e., benign) (9). Our results support this hypothesis. We demonstrate that a metastatic clone (HM-2) isolated from the murine UV-2237 fibrosarcoma was less stable for metastasis than a LM clone (LM-1) isolated from the same tumor (Fig. 1). When the HM-2 clone was subcloned immediately after recovery from frozen stock, all of its subclones exhibited metastatic behavior similar to that of their parent line. However, subclones isolated from the HM-2 clone after its continuous growth *in vitro* and *in vivo* for 60 days varied significantly among themselves (up to 140-fold) and from the parent HM-2 clone. The development of metastatic diversity in the HM-2 clone was not unidirectional. Some of the HM-2 subclones were more metastatic, but others were dramatically less metastatic than the parent clone.

The degree of heterogeneity for metastasis that we found with the propagated HM-2 clone (from a median of 2 to 280 metastases per mouse) was very similar to that originally observed with the unselected parent UV-2237 tumor (14). The fact that the HM clone did not show a unidirectional progression is compatible with the hypothesis that a genetic mechanism plays a role in the process. An increased rate of mutation, in the absence of selection pressures, could lead to the emergence of less malignant, as well as more malignant, variants. Evidence for this concept comes from mutagenesis experiments on different murine neoplasms. Mutagenesis of a malignant teratocarcinoma cell line or the Lewis lung carcinoma cell line resulted in the appearance of variant clones incapable of progressive growth in normal syngeneic recipients (25, 26). Also, mutagenesis of an uncloned population of mouse fibrosarcoma cells led to the emergence of variants with increased malignant potential (24), whereas the mutagenesis of mouse sarcoma cells led to variants with malignant properties less than or equal to the parental tumor (27).

The growth of tumor cells in a subcutaneous space does not select for a metastatic phenotype (1–3, 11). This could explain why we did not observe any differences in the degree or the direction of variability between the HM-2 clone propagated *in vitro* and that propagated *in vivo*. These results also agree with a recent observation that cloned populations of the mouse B16 melanoma are not stable for expression of their metastatic phenotype, whether propagated in culture or by serial subcutaneous passage (28).

A possible explanation for the finding that the UV-2237 LM-1 clone is more stable for the phenotype of metastasis than the UV-2237 HM-2 clone is that the HM clone, being more "progressive," is less stable genetically than the less "progressive"

LM clone. The data shown in Tables 1 and 2 support this suggestion. We studied the rate of mutation in four pairs of LM and HM tumor cell lines isolated from three different murine tumors. In two of the three tumor systems that we used (UV-2237 and K-1735), the LM and HM clones arose spontaneously (14–16). In the third tumor system, the HM line of the SF-19 fibrosarcoma was obtained after multiple *in vitro* treatments of the parent SF-19 tumor with mutagenic doses of UV radiation (18, 24). In all of these systems, however, an increased rate of mutation was found to be associated with increased malignancy (metastasis).

Our studies demonstrate that metastatic cells have a higher spontaneous mutation rate than nonmetastatic cells isolated from the same neoplasm. This suggests that a genetic mechanism may be responsible for the process of tumor progression, but it does not rule out the possibility that epigenetic factors also may influence the process (8, 12, 13). Given the complex relationship between host and tumor, it is probably unlikely that processes such as tumor progression or metastasis, or both, can be explained by a single mechanism (29). However, our result supports the concept that mutational events can be responsible for the evolution and progression of at least some tumors (24–27).

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1. Fidler, I. J. & Kripke, M. L. (1977) *Science* **197**, 893–895.
2. Fidler, I. J. (1978) *Cancer Res.* **38**, 2651–2660.
3. Poste, G. & Fidler, I. J. (1980) *Nature (London)* **283**, 139–146.
4. Reddy, A. L. & Fialkow, P. J. (1980) *J. Exp. Med.* **150**, 878–886.
5. Fialkow, P. J. (1976) *Biochim. Biophys. Acta* **458**, 283–310.
6. Fialkow, P. J. (1972) *Adv. Cancer Res.* **15**, 191–226.
7. Foulds, L. (1954) *Cancer Res.* **14**, 337–339.
8. Klein, G. & Klein, E. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 2121–2125.
9. Nowell, P. C. (1976) *Science* **194**, 23–28.
10. Loeb, L., Springate, C. F. & Battula, N. (1974) *Cancer Res.* **34**, 2311–2321.
11. Goldberg, S. & Defendi, V. (1979) *Somatic Cell Genet.* **5**, 887–895.
12. Barrett, C. J. & Ts'o, P. O. P. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3761–3765.
13. Prehn, R. T. (1976) *Adv. Cancer Res.* **23**, 203–236.
14. Kripke, M. L., Gruys, E. & Fidler, I. J. (1968) *Cancer Res.* **38**, 1962–1967.
15. Kripke, M. L. (1979) *J. Natl. Cancer Inst.* **63**, 541–548.
16. Fidler, I. J., Gruys, E., Cifone, M. A., Barnes, Z. & Bucana, C. (1981) *J. Natl. Cancer Inst.*, in press.
17. Fidler, I. J. & Kripke, M. L. (1980) *Cancer Immunol. Immunother.* **7**, 201–205.
18. Cifone, M. A. & Fisher, M. S. (1980) *J. Cell Biol.* **87**, 290 (abstr.).
19. Fidler, I. J. (1978) *Methods Cancer Res.* **15**, 399–439.
20. Siegal, S. (1956) *Nonparametric Statistics for the Behavioral Sciences* (McGraw Hill, New York), pp. 116–127.
21. Luria, E. S. & Delbrück, M. (1943) *Genetics* **28**, 491–511.
22. Cifone, M. A., Kripke, M. L. & Fidler, I. J. (1979) *J. Supramol. Struct.* **11**, 467–476.
23. Baker, R. M., Brunette, D. M., Mankovitz, R., Thompson, L. H., Whitmore, G. E., Siminovich, L. & Till, J. E. (1974) *Cell* **1**, 9–21.
24. Fisher, M. S. & Cifone, M. A. (1981) *Cancer Res.*, in press.
25. Boon, T. & Van Pel, A. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1519–1523.
26. Van Pel, A., Georlette, M. & Boon, T. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5282–5285.
27. Kerbel, R. S. (1979) *Am. J. Pathol.* **97**, 609–622.
28. Poste, G., Doll, J. & Fidler, I. J. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 6226–6230.
29. Potter, V. R. (1978) *Br. J. Cancer* **38**, 1–23.