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The Origin of Metastatic Heterogeneity in Tumors*

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Abstract—*These studies were conducted to determine whether the metastatic heterogeneity that is frequently observed in primary neoplasms is a consequence of multicellular transformation or acquired genetic variability. BALB/c embryo fibroblasts were infected* in vitro *with mouse sarcoma virus. Six tumor colonies, each derived from a single transformed cell, were isolated and propagated as individual cell lines. Twenty-four days after virus infection, mice were injected s.c. or i.v. with viable cells harvested from the individual lines. Subcutaneous tumors developed in nearly all of the mice and regressed* 30 days *after inoculation. In contrast, the production of lung tumor colonies varied significantly among the cell lines. Moreover, the individual lines were found to be heterogeneous. This conclusion is based on results of experiments in which two cell lines exhibiting either a low or high propensity to produce lung tumor colonies were subcloned. Cells from these subclones were injected i.v. into syngeneic mice. The subclones differed significantly among themselves and from the parent culture in their ability to produce lung tumor colonies. We conclude that regardless of whether neoplasms are unicellular or multicellular in origin, they can be heterogeneous and contain subpopulations of cells with different metastatic properties by the time of diagnosis.*

INTRODUCTION

NEOPLASMS are composed of subpopulations of cells that have a wide range of phenotypes. Cells obtained from individual human and animal tumors have been shown to differ with respect to their immunogenicity [1–3], antigenicity [4–6], growth rate [7], radiosensitivity [8], karyotypes [9], pigment production [10], hormone receptors [11] and susceptibility to cytotoxic drugs [12–16]. In recent years it has become evident that the metastatic phenotype also displays a heterogeneous profile. Malignant tumors are not populated by cells of equal metastatic potential but consist of subpopulations of cells with widely differing metastatic capacities [17–23].

The source of this cellular diversity may be dependent upon the origin of the tumor. Those neoplasms that have a multicellular origin, for example, some chemically induced tumors [24], may exhibit cellular heterogeneity because they are populated with progeny of several transformed cells. However, the source of diversity is less apparent in those tumors that a unicellular origin [25, 26]. Nowell [27] has hypothesized that heterogeneity may occur in unicellularly derived neoplasms as a consequence of acquired genetic variability within developing cells (clones). This genetic variability, coupled with the selection pressure exerted by host mechanisms, leads to the emergence of new sublines with an increased potential for survival that is manifested by enhanced malignancy. Therefore, regardless of whether its origin is multicellular [24] or unicellular [25, 26], the primary neoplasm generally consists of diverse subpopulations of cells by the time of clinical presentation.

Previously, in our studies of the metastatic heterogeneity of murine neoplasms, we have used tumor systems of relatively long duration [17, 18]. In the study reported here we have attempted to determine the origin of metastatic heterogeneity in a murine tumor obtained by the viral transformation of normal fibroblasts, which enabled us to examine metastatic characteristics within a few weeks of tumor induction.

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MATERIALS AND METHODS

Mice

Specific-pathogen-free mice of the inbred strain BALB/cAnN (BALB/c) were supplied by the Frederick Cancer Research Center's Animal Production Area.

Culture conditions

All cell cultures were grown in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and 2-fold-concentrated vitamin solution, the components of which were obtained from Flow Laboratories, Rockville, MD. No antibiotics were included in the media used for routine maintenance of the cell lines. All cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. All cell lines were tested for and found to be free of *Mycoplasma*.

For *in vivo* studies, the tumor cells were harvested from subconfluent cultures in exponential growth phase by overlaying the cells with a thin layer of 0.25% trypsin–0.2% EDTA for 1 min. The flask was tapped sharply to dislodge the cells and the supplemented Eagle's minimal essential medium was added immediately. The cells were then washed and resuspended in Hanks' balanced salt solution (HBSS). Tumor cell viability was about 95%, based on the ability of the cells to exclude trypan blue. Only suspensions composed of single cells were used for i.v. injection into animals.

Infection of BALB/c embryo fibroblasts with mouse sarcoma virus (MSV)

BALB/c embryo fibroblasts were seeded into 100 mm tissue culture dishes (Falcon Plastics, Oxnard, CA) at a dilution calculated to yield 30–40 colonies per dish. The cultures were incubated at 37°C for 2 hr, at which time single cells were identified with an inverted microscope and their position marked. The BALB/c fibroblasts were infected with MSV by titering 10-fold dilutions of supernatants from FG-10 cultures to obtain a limited dilution that transformed a single colony. The FG-10 producer line is a 3T3 cell line of BALB/c lineage originally transformed with cloned MSV and is a non-murine leukemia producer. Nine to ten days after the BALB/c embryo fibroblasts were infected with MSV, six colonies originating from single cells were isolated and harvested using a cloning ring. These colonies were propagated as six individual cell lines (designated as colonies 1–6), which at day 24 after initial infection with MSV were harvested and frozen at −180°C.

Cells from colonies 1 and 2 were cloned at this time exactly as described previously [17], and several of these cloned lines were established and frozen as described above. These cloned cell lines and the cell lines from the original six colonies were taken from the freezer at the same time for use in all *in vivo* experiments.

In vivo *experiments*

Cell lines taken from the freezer at the same time were grown in monolayer culture and harvested (see above). Tumor cell suspensions were diluted in HBSS to yield 1×10^6 cells/ml (>90% viability as assessed by trypan blue exclusion). Six-week-old BALB/c mice were injected i.v. in the tail with 0.2 ml (200,000 cells) or s.c. with 5×10^5 viable cells. The animals that were injected i.v. were killed 21 days later; the lungs were removed, rinsed in water and fixed overnight in Bouin's solution. This procedure allowed identification of sarcoma foci as white areas on a yellow background of uninvolved lung tissue. The number of tumor foci was determined by counting the surface metastases under a dissecting microscope. The mice that were injected s.c. were examined twice weekly, and tumor incidence and size were recorded.

Statistical analysis

All data were analysed by nonparametric statistical methods [28] as detailed in the Results section.

RESULTS

Subcutaneous growth of MSV-transformed BALB/c embryo fibroblasts

In the majority of mice inoculated s.c. with 5×10^5 viable cells, all of the six originally derived colonies produced tumors at the site of injection (Table 1). These tumors were regressor in that, by 30 days after inoculation, none were palpable. There were no obvious differences among any of the tumor colonies with regard to either tumor induction time or growth rate.

Lung colony assay

Although the s.c. growths that resulted from the injection of MSV-transformed fibroblasts were regressor, they were also neoplastic inasmuch as the i.v. injection of the same cells

Table 1. The s.c. growth of MSV-transformed BALB/c embryo fibroblasts in BALB/c mice

Source of cells	Tumor incidence at days after s.c. injection* 7	14	21	30
Colony 1	4/5 (0.2)†	3/5 (0.3)	0/4	0/4
Colony 2	4/5 (0.4)	4/5 (0.6)	3/5 (0.5)	0/5
Colony 3	4/5 (0.4)	4/5 (0.4)	2/5 (0.1)	0/5
Colony 4	3/5 (0.3)	4/5 (0.4)	1/5 (0.1)	0/5
Colony 5	3/5 (0.2)	4/5 (0.5)	1/5 (0.1)	0/5
Colony 6	3/5 (0.3)	4/5 (0.3)	0/5	0/5
Untransformed 3T3 cells	0/5	0/5	0/5	0/5

*Number of mice with tumors/number of mice inoculated; 5×10^5 viable cells/mouse.

†Number in parentheses, mean tumor size in cm; single measurement of long axis with vernier caliper.

produced grossly evident pulmonary nodules (Table 2, Fig. 1). These data were analysed by the Kruskal–Wallis test (2-tailed), which revealed that the numbers of lung nodules resulting from the injection of equal numbers of cells were significantly different among the various colonies ($P \leqslant 0.05$ for 1 vs 5, 6; 2 vs 3,5,6; 4 vs 5; $P \leqslant 0.001$ for 1 vs 2,3,4; 3 vs 4; 4 vs 6). Using Dunn's approximation for multiple comparisons [28], we found that the greatest differences were between colonies 1 and 2 (Ru–Rv = 31.44) and between colonies 1 and 4 (Ru–Rv = 35.67). Thus, the injection of equal numbers of transformed cells derived from different single colonies produced tumors with high metastatic capacity, as assayed by lung colony formation.

In order to examine the degree of heterogeneity found within a single colony, we cloned the two cell lines, colony 1 (low metastatic capacity) and colony 2 (high metastatic capacity) and assessed them by the lung colony assay. As shown in Tables 3 and 4, there were marked differences among the clones with regard to their ability to produce experimental metastases. Application of the Wilcoxon two sample test to the data reported in Table 3 showed that there were significant differences in the median number of lung tumor nodules ($P \leqslant 0.001$) between the parent colony and the clones 4, 11, 23, 24, 28, 29 as well as the untransformed 3T3 cells; significant differences ($P \leqslant 0.02$) were also observed between the parent colony and the clones 5, 6 and 13. At this level there were no differences between the parent colony and the clones 8 and 27. By the Kruskal–Wallis test, as applied to all 13 groups, the possibility that all groups were the same was not likely (Kruskal–Wallis statistic = 66.72035, $P = 0.000000001$). Similar analysis of the data shown in Table 4 revealed that the median number of lung tumors produced by parent colony 2 was significantly different from clones 1, 5, 8, 11 and 14 ($P \leqslant 0.05$), and the Kruskal–Wallis test (statistic = 55.14096) as applied to all groups showed

Table 2. Number of pulmonary metastases produced in BALB/c mice following the i.v. injection of 200,000 viable cells from various MSV-transformed BALB/c fibroblasts

Source of cells	Number of pulmonary metastases*	Median (range)
Colony 1	0, 1, 2, 2, 2, 3, 6, 7, 12, 13	2.5 (0–13)
Colony 5	5, 6, 6, 6, 9, 9, 15, 34, 78, 89	9 (5–89)
Colony 3	10, 12, 13, 14, 18, 19, 20, 21, 30, 57	18.5 (10–57)
Colony 6	1, 3, 9, 13, 24, 25, 33, 35, 37	24 (1–37)
Colony 2	13, 14, 25, 34, 36, 48, 53, 91, 104	36 (13–104)
Colony 4	12, 30, 33, 48, 62, 68, 101, 144, 159	62 (12–159)

*Mice were killed 21 days after i.v. injection of cells. Pulmonary metastases were counted with the aid of a dissecting microscope.

Table 3. Number of pulmonary tumor nodules resulting from the i.v. injection of 2×10^5 MSV-transformed fibroblasts from colony 1 and its cloned subpopulation

Source of cells	Number of pulmonary metastases*	Median (range)
Parent colony 1	2, 3, 6, 8, 8, 10, 14, 18, 19, 22	9 (2–22)
Clone 23	0, 0, 0, 0, 0, 0, 1, 1, 2, 5	0 (0–5)
Clone 28	0, 0, 0, 0, 0, 0, 0, 1, 1, 2	0 (0–2)
Clone 19	0, 0, 0, 0, 0, 1, 2, 2, 2, 3	0.5 (0–3)
Clone 24	0, 0, 0, 0, 0, 1, 1, 2, 2, 3	0.5 (0–3)
Clone 11	0, 0, 0, 0, 0, 2, 2, 2, 3, 5	1 (0–5)
Clone 4	0, 0, 0, 1, 1, 2, 3, 3	1 (0–3)
Clone 13	0, 0, 1, 1, 2, 2, 3, 3, 4, 6	2 (0–6)
Clone 6	1, 1, 2, 2, 2, 2, 3, 3, 4, 5	2 (1–5)
Clone 27	0, 2, 2, 3, 3, 4, 5, 8, 8, 15	3.5 (0–15)
Clone 8	2, 2, 3, 4, 5, 5, 36, 40, 63	5 (2–63)
Clone 5	0, 0, 1, 3, 5, 5, 6, 6, 9	5 (0–9)
Untransformed 3T3 cells	0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0

*Mice were killed 21 days after i.v. injection of cells. Pulmonary metastases were counted with the aid of a dissecting microscope.

Table 4. Number of pulmonary tumor nodules resulting from the i.v. injection of 2×10^5 MSV-transformed fibroblasts from colony 2 and its cloned subpopulations

Source of cells	Number of pulmonary metastases*	Median (range)
Parent colony 2	0, 1, 1, 2, 2, 3, 20, 20, 28, 61, 64, 80, 85, 87, 91	28 (0–3)
Clone 8	0, 0, 1, 1, 1, 1, 2, 2, 3, 3	1 (0–3)
Clone 1	0, 0, 0, 0, 1, 1, 2, 2, 2, 12	1 (0–12)
Clone 5	0, 1, 1, 1, 1, 2, 3, 3, 3, 19	1.5 (0–19)
Clone 14	0, 0, 0, 0, 2, 3, 5, 9, 16, 19	2.5 (0–19)
Clone 11	0, 0, 0, 1, 2, 4, 6, 11, 12, 20	3 (0–20)
Clone 15	0, 2, 3, 3, 4, 4, 7, 7, 17, 18	4 (0–18)
Clone 1	0, 1, 3, 4, 5, 8, 13, 24, 31, 40	6.5 (0–40)
Clone 2	0, 1, 2, 3, 7, 9, 19, 25, 66, 82	8 (0–82)
Clone 13	0, 2, 3, 4, 8, 10, 14, 24, 28, 30	9 (0–30)
Clone 4	0, 0, 4, 4, 11, 11, 29, 35, 37, 38	11 (0–38)
Clone 9	0, 3, 4, 4, 11, 12, 19, 20, 37, 42	11.5 (0–42)
Clone 12	1, 3, 4, 5, 6, 13, 18, 19, 21, 23, 61	13 (1–61)
Clone 6	2, 6, 13, 16, 18, 40, 45, 57, 74	18 (2–74)
Clone 7	3, 14, 16, 18, 33, 57, 59, 62, 64	33 (3–64)
Clone 10	5, 6, 8, 9, 38, 38, 48, 51, 55	38 (5–55)

*Mice were killed 21 days after i.v. injection of cells. Pulmonary metastases were counted with the aid of a dissecting microscope.

that it was not likely that all groups were the same ($P = 0.000002$).

DISCUSSION

The issue we address in the work reported here is whether the metastatic heterogeneity that frequently is observed in primary tumors [17–23] is a consequence of multicellular transformation [24] or acquired genetic variability [27]. From our data it appears that, at least with the system used here, both possibilities could contribute to this metastatic diversity.

The cells derived from the six originally transformed colonies exhibited marked differences in experimental metastatic capacity as assessed by the lung colony assay. Thus, the i.v. injection of 2×10^5 viable cells from colony 1 into syngeneic BALB/c mice produced a median number of 2.5 lung nodules after 21 days, whereas the injection of equal numbers

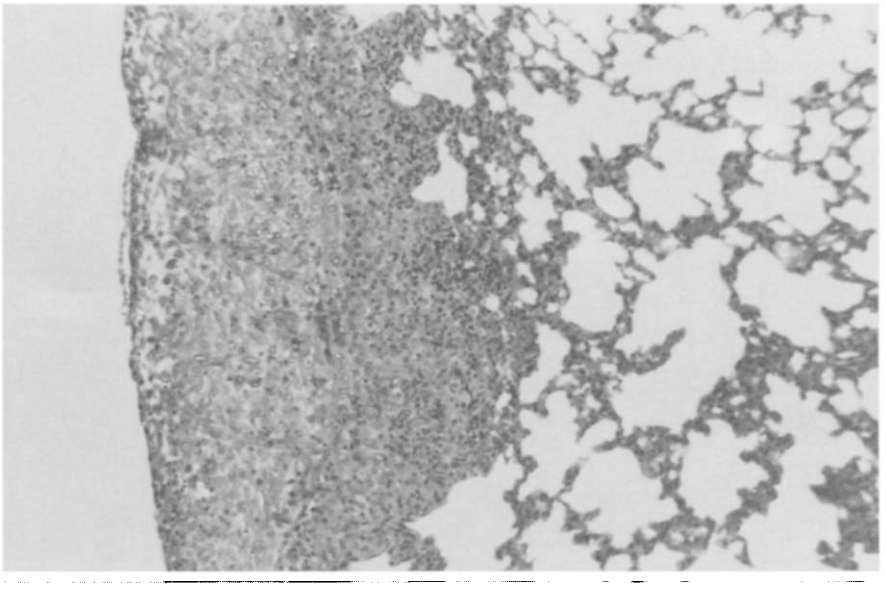

Fig. 1. *A lung nodule appearing three weeks after injection of MSV-transformed fibroblasts into BALB/c mice* (H & E × 160).