Metastatic Potential of B16 Melanoma Cells after In Vitro Selection for Organ-specific Adherence

PETER A. NETLAND* and BRUCE R. ZETTER*‡
*Department of Physiology and Biophysics, Harvard Medical School, Boston, Massachusetts 02115; and ‡Department of Surgery, The Children's Hospital and Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT Heterogeneous primary tumors contain subpopulations of cells that differ in ability to metastasize to specific host organs. We have used cryostat sections of host organs to select for metastatic variants of B16 melanoma cells with increased adhesion to specific syngeneic tissues. By repeating the selection procedure with lung tissue, a subpopulation of cells was isolated that demonstrated a specific increase in binding to cryostat sections of mouse lung. This altered binding was reflected by a sixfold increase in the frequency of lung metastasis 21 d after tail vein injection of the tumor cells. In contrast, B16 melanoma cells selected on cryostat sections of mouse brain showed no increase in adhesion to brain or lung tissue and the metastatic pattern in vivo was not significantly different compared with the parent cell line. When cells selected for increased adhesion to cryostat sections of lung were further examined in vitro, they showed altered morphology and increased motility but no change in growth rate. These results demonstrate that alterations in the adhesive interactions between metastatic tumor cells and a specific host tissue can directly affect the frequency of metastasis to that tissue in vivo.

During the process of hematogenous metastasis, tumor cells enter the bloodstream, arrest in the vasculature of a distant organ, and extravasate into the tissue space (1, 2). Although tumor cell arrest can be mediated by nonspecific trapping, there is increasing evidence for specific adhesive interactions between tumor cells and vascular components (2–5). At least two roles have been proposed for these specific interactions. First, such interactions may promote the passage of tumor cells through the vessel wall and thereby increase the efficiency and frequency of metastasis. Second, organ-specific adhesive interactions may have a role in determining the preferential colonization of one or more organs by certain metastatic tumor cells.

Subpopulations of tumor cells can be isolated that demonstrate different metastatic potential to specific host organs (2, 6). Such subpopulations have been identified in parent tumor cell populations that exhibit considerable cellular heterogeneity. Typically, tumor cell subpopulations have been isolated by selection in vivo or by selection in vitro of random clones from parent cell lines. These procedures yield tumor cell subpopulations that demonstrate varying ability to complete one or more of the many steps involved in the metastatic process.

To study the role of cell adhesion in organ-specific metastasis, we and others have used a cryostat section binding assay that was originally devised to study lymphocyte adherence to specific host blood vessels (7, 8). With this assay, it can be clearly shown that certain tumor cells bind preferentially to cryostat sections of the tissues that they colonize in vivo (9, 10). These experiments identify a correlation but not a causative relationship between tissue-specific adhesive interactions and the eventual site of tumor metastasis.

To examine the hypothesis that specific adhesive interactions influence the site and frequency of tumor metastasis, we have attempted to select subpopulations of tumor cells that display altered binding to cryostat sections from certain host tissues. Using this approach, we find that tumor cell subpopulations can be isolated in vitro on the basis of their selective binding to specific tissues. The changes in organ-specific binding are accompanied by changes in tumor cell morphology and motility. Furthermore, cells selected for organ-specific adhesion in vitro displayed changes in organ-specific metastatic colonization in vivo. These results provide new evidence for a critical role of specific cell–tissue adhesion interactions in organ-specific metastasis.

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MATERIALS AND METHODS

Selection of Variant Cell Lines: Fresh, unfixed tissues obtained from 5–10-wk-old C57BL/6 mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) were embedded in Tissue Tek II O.C.T. compound (Lab-Tek Division, Miles Laboratories Inc., Naperville, IL) and frozen at −30°C. Cryostat sections (10 μm) were prepared using an Ames cryostat (model 4550, Miles Ames Div., Miles Laboratories, Inc., Elkhart, IN) and placed on 1.2-cm coverslips (Bellco Glass, Inc., Vineland, NJ) in wax pencil circles. To sterilize the cryostat sections, the coverslips were placed 5 in. under 2 × 15-W General Electric germicidal lights (General Electric Co., Cleveland, OH) and exposed for 5 min at 5–7°C, then covered in a sterile petri dish. This dose of ultraviolet light was bactericidal as determined by a germicidal lamp monitor (Vangard International, Inc., Neptune, NJ). A single-cell suspension of B16-F1 melanoma cells (provided by Dr. Isaiah J. Fidler, M. D. Anderson Hospital and Tumor Institute, Houston, TX) was prepared with 0.1% trypsin (type III, Sigma Chemical Co., St. Louis, MO) in calcium- and magnesium-free phosphate-buffered saline (PBS), washed extensively with 0.5% soybean trypsin inhibitor (Gibco Laboratories, Grand Island, NY) in PBS, and washed once with Dulbecco's modified Eagle's medium (DME).[1] Cells (7×10^5 in 100 μl of medium per section) were incubated for 30 min at 5–7°C on the sterile cryostat sections with agitation on a Tek-Tator V rotator (American Dade, Miami, FL) at 70 rpm. After incubation, the medium was absorbed with sterile gauze, and the coverslips were rinsed gently with cold (4°C) PBS. The tissue sections and remaining adherent cells were taken up in warm (37°C) DME containing 10% calf serum and placed in tissue culture dishes. The cells adhering to tissue from three coverslips were pooled and plated in a Nunc 35-mm culture dish (Vangard International, Inc.). After incubation for 16 h, the medium was changed. Triplicate cultures were prepared at each selection step, propagated in vitro, and assayed for binding to cryostat sections as described below. Cultures that demonstrated maximal binding to a desired tissue and minimal binding to other tissues were subjected to further selection on cryostat sections derived from that tissue. B16-L4 melanoma cells, for example, were isolated after four selection steps using cryostat sections of lung.

Adherence Assay: To quantitate the in vitro binding of tumor cells to specific tissues, tumor cells were incubated on fresh cryostat sections as described (9). Briefly, tumor cells were labeled with 20 μCi/ml $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA) for 16 h in DME supplemented with 10% calf serum, a single-cell suspension was prepared, and labeled cells were incubated for 40 min on cryostat sections as described above. After the incubation, excess medium was removed by touching the edge of the coverslip with adsorbant tissue, and the coverslips were placed on edge in PBS containing 1% glutaraldehyde for 15 min and then rinsed gently with PBS. The amount of radioactivity associated with the sections was measured in a Gamma Trac 1191 gamma counter (Tracor Analytic Inc., Elk Grove, IL), and the number of cells bound to each section was calculated. Typically, 0.1–4% of the cells added initially remained bound after rinsing. The relative adherence ratio (8) is the ratio of the number of adherent sample cells to the number of adherent BALB/c mouse 3T3 cells that bound to duplicate sections.

Experimental Metastasis Assay: Single-cell suspensions of trypsinized tumor cells were prepared as described above, washed, and resuspended in 0.15 M NaCl at a concentration of 2×10^6 cells/ml. C57BL/6 mice were injected with 0.15 ml of these suspensions in a lateral tail vein. At necropsy 21 d later, organs were removed, and the number of surface metastases was determined without magnification.

Cell Culture: Tumor cells were grown in DME supplemented with 10% calf serum (Gibco Laboratories, Grand Island, NY), 2 mM glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin.

To compare morphology of tumor cell lines, we examined cells by phase-contrast microscopy 2 d after cells were plated at 5×10^4 cells per 25 cm^2 flask (Falcon Labware, Oxnard, CA).

To assess the growth rate of tumor cells, we counted cells at 2-d intervals after plating at 10^4 cells/well in 12-well dishes (Costar, Cambridge, MA). Cells were trypsinized and counted with a Coulter counter.

Cell Motility Studies: Phagokinetic track areas were determined as previously described (11, 12), with the following modifications. Before coating with gold particles, 22 × 22-mm glass coverslips were dipped in 1% BSA (fatty acid- and globulin-free, Sigma Chemical Co.) and dried or incubated with 20 μg/ml bovine fibronectin (Calbiochem-Behring Corp., La Jolla, CA) for 30 min and used without drying. After coating the coverslips with gold particles, tumor cells (3,000) were incubated on the coverslips in DME containing 10% calf serum for 17 h and fixed in 10% buffered formalin phosphate (Fisher Scientific Co., Pittsburgh, PA). Phagokinetic track areas were measured by transferring the image from the inverted microscope (10× objective) to an Omnicon FAS-II image analysis system (Bausch & Lomb Inc., Rochester, NY). Because cell divisions or cell collisions may alter track areas, only tracks formed by a single cell were analyzed.

Coverslips were also examined with a Nikon MS inverted microscope (Nikon Inc., Garden City, NY) under incident light from a MK II fiber optic light (Ehrenreich Photo-Optical Industries, Inc., Garden City, NY). Phagokinetic tracks were photographed with a Nikon microflex HFM photomicrographic attachment.

RESULTS

We have used cryostat sections of different organs to select and isolate B16 melanoma cell subpopulations that bound to specific tissues. When the adherent cells were seeded in a 35-mm culture dish, they reached confluence within 1 wk. Using this procedure, we attempted to select subpopulations of the B16-F1 melanoma cell line that had increased adhesivity to mouse lung and brain.

The organ-specific adherence of the selected cell lines was determined by measuring the binding of ^{51}Cr-labeled tumor cells to fresh cryostat sections from the tissues of interest. As shown in Fig. 1*a*, in vitro selection for adherence to lung

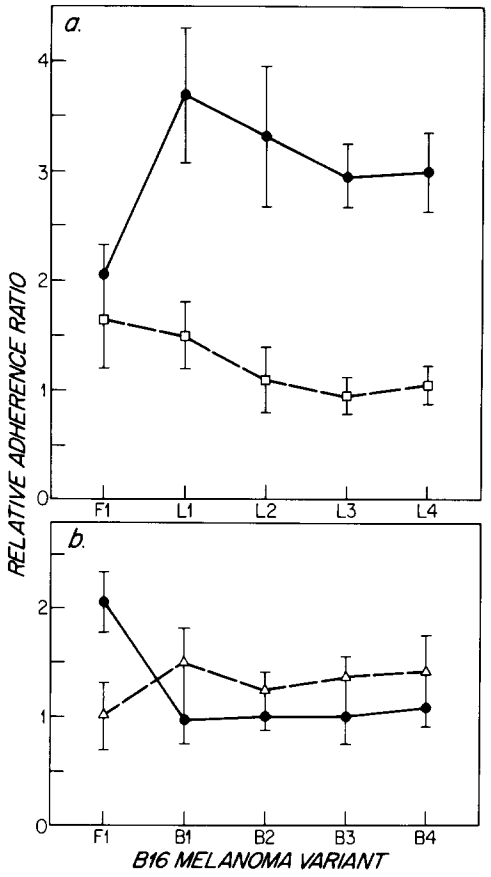

FIGURE 1 In vitro binding of variant cell lines selected for adherence to cryostat sections. Adherent cells were selected using cryostat sections of lung (*a*) or brain (*b*) as described in Materials and Methods. After each selection step, variant cell lines were labeled with ^{51}Cr and incubated for 40 min at 5–7°C with gentle agitation on fresh 10-μm cryostat sections of lung (●), liver (□), or brain (△) obtained from C57BL/6 mice. After washing, the radioactivity associated with individual sections was counted to determine the ratio of the number of adherent sample cells to the number of BALB/c mouse 3T3 cells that bound to duplicate sections. Data points are means ± standard deviations for at least five samples.

[1] *Abbreviation used in this paper:* DME, Dulbecco's modified Eagle's medium.

sections resulted in cell lines that demonstrated increased binding to lung tissue in vitro. In contrast with these results, it was not possible to select populations that demonstrated changes in binding to cryostat sections of mouse brain (Fig. 1 *b*) or testis (not shown).

In general, the cell lines showed increased adhesivity only to the tissues on which they were selected. In some cases, a cell line that was selected for increased binding to one tissue showed decreased binding, relative to the parental population, to sections prepared from other tissues. A subpopulation selected on cryostat sections of lung, for example, demonstrated reduced binding to liver sections (Fig. 1 *a*). Similarly, a cell line produced by selection on cryostat sections of brain demonstrated a marked decrease in binding to lung (Fig. 1 *b*). This demonstrates that both positive and negative selection for cell adhesivity can be achieved using these methods without causing nonspecific changes in overall cell adhesiveness.

We next examined the in vivo colonization patterns of the sublines that had been selected for altered adhesion to specific tissues. The tumor cells were injected intravenously via the tail vein, and the number of visible surface metastases in the organs of interest were determined after 21 d. The ability to colonize the lung was increased significantly after one selection step on cryostat sections of lung, whereas the ability to colonize the liver did not change (Table I). Because the metastatic potential of the parent B16-F1 melanoma cell line was not altered over at least 20 passages in vitro (not shown), these changes did not result simply from repeated passages in culture. Fig. 2 shows that the B16-L4 melanoma cell line, selected for increased adherence to lung tissue, showed a sixfold increase in the number of lung metastases compared with the parental B16-F1 melanoma cell line. In contrast, a cell line selected on cryostat sections of mouse brain (B16-B4) showed no significant change in the in vivo metastatic pattern. This indicates that the increase in metastatic frequency in the lung is dependent upon the specific tissue used for the selection procedures.

TABLE 1. *Experimental Metastasis Potential of Variant Cell Lines Selected for Adherence to Cryostat Sections of Lung*

Cell line	Pulmonary metastases	Hepatic metastases
	mean ± SD	
B16-F1 (*n* = 8)	19 ± 13	0
B16-L1 (*n* = 7)	64 ± 14	0.4 ± 0.5
B16-L2 (*n* = 4)	81 ± 12	0
B16-L3 (*n* = 7)	128 ± 30	0.3 ± 0.8
B16-L4 (*n* = 9)	115 ± 26	0.3 ± 0.7

Metastases were counted 21 d after tail vein injection of 3×10^5 trypsinized viable cells into C57BL/6 mice.

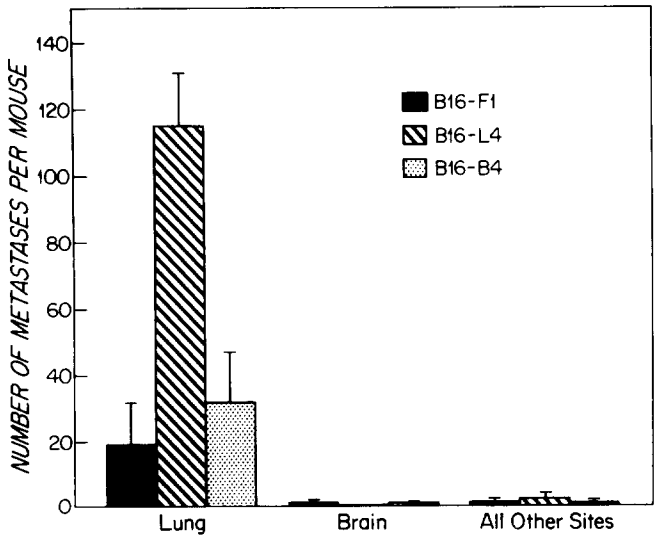

FIGURE 2 Experimental metastasis potential of B16 melanoma cell lines selected for adherence to cryostat sections. Metastases were counted 21 d after tail vein injection of 3×10^5 trypsinized viable cells into groups of at least 8 C57BL/6 mice. *All other sites* refers to metastases found in organs other than lung and brain, including kidney, adrenal, liver, mesentery, omentum, inferior vena cava, and submaxillary gland. Values are means ± standard deviations.

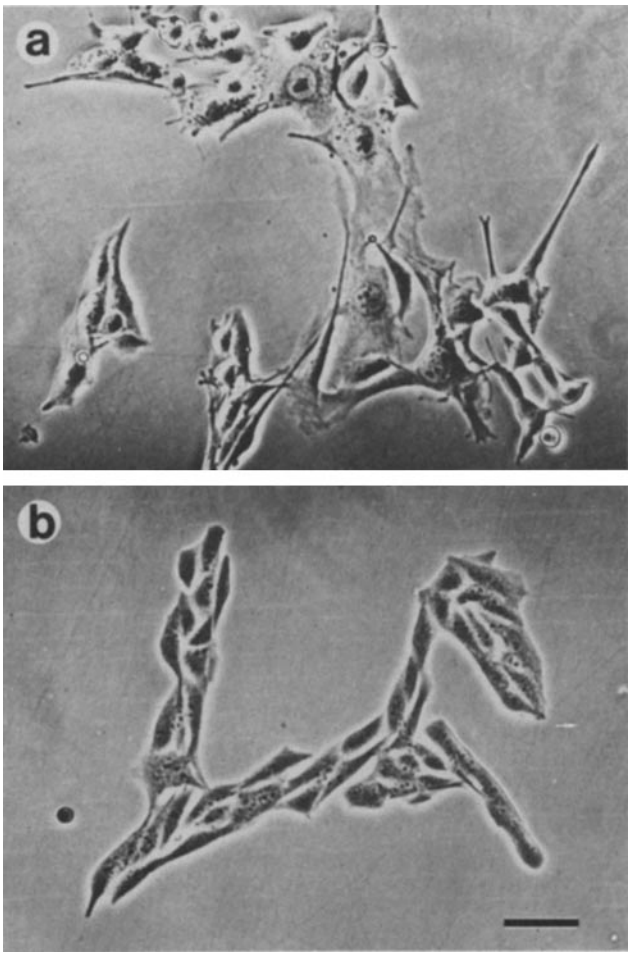

FIGURE 3 Morphology of B16 variant selected for adherence to cryostat sections of lung (B16-L4). After plating at sparse density, cells were incubated for 2 d and examined by phase-contrast microscopy. B16-F1 (*a*) and B16-L4 (*b*) melanoma cells exhibited distinct morphological differences. Bar, 60 μm. × 165.

Further Characterization of B16 Melanoma Cell Line Selected on Cryostat Sections of Mouse Lung (B16-L4)

The B16-L4 melanoma cell line that showed increased adhesion to lung tissue in vitro as well as increased lung colonization in vivo was further examined for its morphological, growth, and motility characteristics. As shown in Fig. 3, the B16-L4 melanoma cells were more homogeneous in shape and had fewer extended processes than did the B16-F1 line.

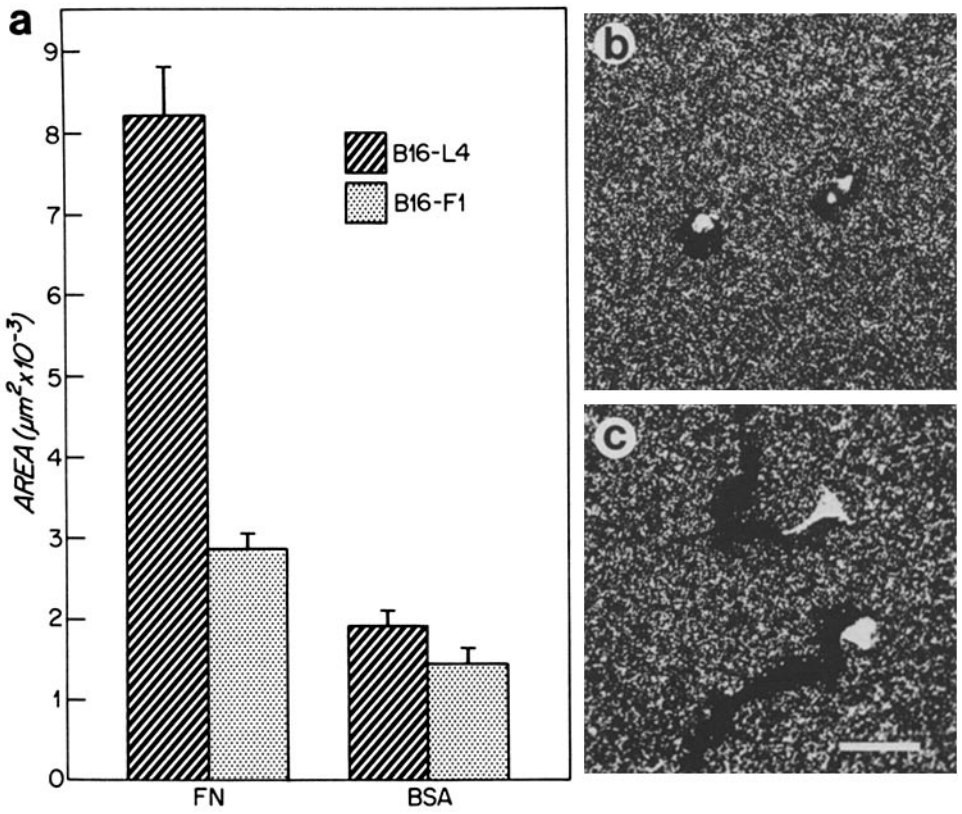

FIGURE 4 Motility of B16-F1 and B16-L4 melanoma cells. Cells were seeded on gold-coated coverslips that had been treated with fibronectin (*FN*) or bovine serum albumin (*BSA*) as described in Materials and Methods. The cultures were incubated at 37°C in DME containing 5% calf serum for 17 h, then fixed with 10% buffered formalin phosphate. Phagokinetic track areas were measured using an Omnicon FAS-II image analysis system (*a*). Values are means ± standard error for 81 tracks. The phagokinetic tracks of B16-F1 (*b*) and B16-L4 (*c*) melanoma cells on fibronectin-treated gold-coated coverslips were observed under incident light with a Nikon MS inverted microscope. Bar, 100 μm. × 105.

When the same cells were assayed for their ability to move across gold-coated coverslips in a quantitative phagokinetic assay, the B16-L4 melanoma cells displayed significantly higher levels of chemokinetic motility in comparison with the parental cell line. This difference was amplified when the cells were seeded on coverslips coated with fibronectin plus colloidal gold (Fig. 4). In contrast with the change in cell motility, the growth rates of the two cell lines were nearly identical when the cells were cultured in medium supplemented with 10% calf serum (Fig. 5). The population doubling time during exponential growth was 23 ± 2 and 22 ± 2 h for B16-F1 and B16-L4 melanoma cells, respectively.

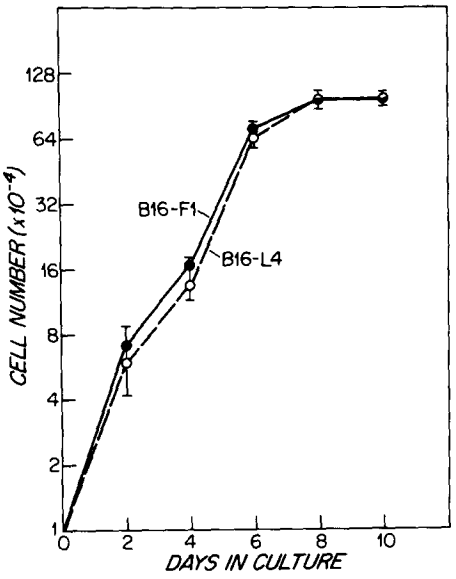

FIGURE 5 Growth rates of B16-F1 and B16-L4 melanoma cells in vitro. Cells were trypsinized and plated in medium containing 10% calf serum. At various intervals, the cells were trypsinized and counted with a Coulter counter. Data points are means ± standard deviations for three samples.

DISCUSSION

Because metastasis is a multistep process, in vivo selection for metastatic variants may yield cells that have been altered in any of the several steps involved in metastasis. One effective approach to investigating the role of specific cellular events in the metastatic process is to determine the metastatic potential of cells that have been previously selected for a specific characteristic in vitro. In contrast with the repeated selection cycles required to select metastatic variants such as the B16-F10 melanoma cell line (13), we found that changes in metastatic potential can be obtained with one selection step using cryostat sections.

We first attempted to isolate subpopulations of the B16-F1 melanoma cell line that displayed altered adhesion to specific tissues. Previously, tumor cell lines have been selected in vitro for certain adherence properties, including altered attachment to plastic (14–16) or collagen (17), reduced homotypic adhesion (18), and decreased binding to immobilized lectins (19). Our experiments were designed to select subpopulations of cells from a heterogeneous population of tumor cells that adhered preferentially to specific tissues. After identifying any changes in organ-specific adherence, we could study the in vivo behavior of the cell lines to examine the effect of their altered adhesivity upon the frequency of metastasis to specific tissues.

When a heterogeneous population of B16-F1 melanoma cells was selected for adherence to lung tissue, we obtained subpopulations that displayed increased adhesion to cryostat sections of lung (B16-L4). It has been demonstrated that, after multiple selections in vivo, B16 melanoma cell lines that

display increased preference for brain colonization (20) show increased organ-specific adhesion to brain endothelial cells in vitro (21). Under the conditions of our selection procedure, however, we were not able to select populations that showed increased adhesion to mouse brain or testis. This may be because there is a low prevalence of such cells in the parent B16-F1 melanoma cell population.

To examine the role of adhesive interactions in the metastatic process in vivo, tumor cell subpopulations selected for altered adherence properties were injected intravenously into mice. The lung-adherent B16-L4 melanoma cells showed a markedly increased and specific metastatic potential to the lung. Compared with the parental B16-F1 melanoma cell line, there was a sixfold increase in the number of visible pulmonary metastases 21 d after tail vein injection of B16-L4 melanoma cells. Because the in vitro isolation procedures selected for a specific cellular characteristic, this indicates an important role for organ-specific adhesive interactions in the metastatic process. In contrast, cell lines selected on cryostat sections of brain did not demonstrate increased metastatic potential to the lung. This indicates that the selection procedure using cryostat sections from other tissues besides lung does not cause increased frequency of lung colonization in vivo.

We also analyzed the B16-L4 melanoma cell line for alterations in cell growth and motility in vitro. No differences in growth rate over a 10-d period could be seen between the B16-F1 and B16-L4 melanoma cell lines. There was, however, a significant increase in the motility of the B16-L4 melanoma cell line as measured in a quantitative phagokinetic assay. The differences in cell motility between the two cell lines were even more pronounced on fibronectin, a substratum that has been shown to mediate haptotactic movements of B16 melanoma cells (22, 23). These results are consistent with the finding of increased motility of a highly metastatic variant from the K-1735 melanoma tumor (24). These results also suggest a correlation between cell-substratum adhesion and cellular motility, which may be important during tumor cell invasion of host tissues (25).

In summary, we have demonstrated that B16 melanoma variant cell lines can be selected for altered adhesion to cryostat sections of specific tissues. Cell lines selected for increased adhesion to lung cryostat sections show increased lung colonization in vivo. These cells also show altered morphology and increased motility, but no change in growth rate in vitro. We conclude that adhesive interactions between metastatic tumor cells and specific host tissues can influence the frequency of metastasis in those tissues in vivo.

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