Development of a Preclinical Model of Spontaneous Human Melanoma Central Nervous System Metastasis

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Abstract

Metastatic spread of melanoma to the central nervous system (CNS) is associated with dismal prognosis. Preclinical testing of novel therapeutic approaches would be aided by the development of appropriate models of spontaneous CNS metastasis arising from primary tumors. A highly metastatic variant of the WM239A human melanoma cell line, designated 113/6-4L, was generated and used to test the efficacy of long-term, low-dose metronomic cyclophosphamide and vinblastine chemotherapy on advanced established metastatic disease in sites such as liver, lungs, and lymph node. This treatment resulted in control of advanced, systemic disease and prolongation of survival. Among long-term surviving mice, 20% showed the presence of spontaneous brain metastases. Two cell lines (131/4-5B1 and 131/4-5B2) were generated from such metastases, which were found to spontaneously metastasize to brain parenchyma with occasional localization to leptomeninges, after orthotopic transplantation and removal of the primary tumor. The cell lines were found to have increased ability to proliferate in brain-conditioned medium and displayed enhanced adhesion to lung and brain endothelial cells. These findings represent the first report of spontaneous CNS metastases generated from primary tumors of any human cancer in mice, which heritably maintains this phenotype, and as such, the variant cell lines generated should aid studies in the biology and treatment of CNS metastases, especially of melanoma origin. [Cancer Res 2008;68(12):4500–5]

Introduction

Metastatic spread of melanoma, especially to the central nervous system (CNS), is associated with a dismal prognosis (1). Present treatment strategies show only low response rates, bring about small increases in median survival, and as a result of poor penetration of the blood-brain barrier, and possibly other factors, have little activity against melanoma CNS metastases as well as brain metastases in other tumor types (2). Presently there is a lack of effective chemotherapeutic options for treatment of brain metastases. The problem of CNS metastases is generally growing in magnitude, particularly for certain malignancies where new therapies that are effective in controlling for a time systemic metastatic disease are currently available (3). As such, the importance of CNS as a sanctuary site for melanoma and other types of cancer is an essential aspect to consider in the development

of more effective therapeutic treatments (4, 5). Clearly, this increases the need for developing appropriate preclinical models of spontaneous CNS metastases in which tumor spread from a primary orthotopic site leads to the spontaneous formation of metastatic lesions in the brain in a manner that reflects the clinical presentation of the disease (6). Such a model would facilitate the study of the biology and treatment of such lesions. We report here the successful development of such a model, which could in principle, be applied to other types of cancers.

Materials and Methods

Generation of highly metastatic melanoma cell line 113/6-4L. One million human melanoma WM239A cells were injected subdermally into four CB-17 severe combined immunodeficient (SCID) female mice. Primary tumors were allowed to develop and resected once they reached a size of 400 to 500 mm³. Mice were sacrificed (4–6 mo posttumor resection) and lungs were examined for the presence of metastases. Lungs from one mouse with evidence of tumor involvement were adapted for tissue culture and cell line 113/6-4L derived. One million 113/6-4L cells were injected and primary tumor was resected as above. Mice were sacrificed at 6 to 8 wk postprimary tumor resection and lungs were examined for the presence of metastases. Extensive metastatic involvement was observed.

Metronomic cyclophosphamide and vinblastine therapy. One million 113/6-4L cells were injected subdermally into CB17-SCID female mice, and primary tumors were resected as above. To examine the efficacy of cyclophosphamide and vinblastine on metastatic melanoma, mice were separated into 4 groups (10 mice per group); group 1, normal saline (NS) daily in drinking water and i.p. injections of NS 3× weekly; group 2, NS daily in drinking water and vinblastine; group 3, NS (i.p. 3× weekly) and cyclophosphamide; group 4, vinblastine and cyclophosphamide. Vinblastine was given at a dose of 0.33 mg/Kg administered by i.p. injection (3× weekly) and cyclophosphamide as 20 mg/Kg daily in drinking water, respectively. Groups 3 to 4 were treated with an initial bolus dose of cyclophosphamide (100 mg/Kg) at day 1 of treatment and repeated every 6 wk, as described by Shaked and colleagues (7). Mice were sacrificed when moribund (usually as a result of breathing difficulties).

Generation of brain metastatic cell lines 131-4-5B1 and 131/4-5B2 and characterization of their brain metastatic potential. The brains of mice showing the presence of melanoma metastases were minced and plated in RPMI 1640 supplemented with 5% fetal bovine serum. Colonies of metastatic melanoma cells were isolated and cultured. A similar approach was taken to derive cell lines from lung metastases and which gave rise to the cell lines 131/7-4L and 131/8-2L. After derivation, cell lines were expanded by three additional passages and aliquots were frozen. Cells used for injection were expanded by three to four additional passages to generate sufficient number of cells for injections. For these experiments, three mice were injected with 113/6-4L cells, four and five mice for 131/7-4L and 131/8-2L, respectively, and seven and six mice for 131-4-5B1 and 131/4-5B2, respectively. Primary tumors were removed when they reached a size of 500 to 700 mm³; mice were sacrificed once moribund, as above. The presence of melanoma metastases in lung, liver, kidney, and brain was examined by means of immunostaining with HMB-45 antibody (Novocastra; 1:30 dilution), a marker for melanoma. For each tissue, up to 5 sections of 50 μm apart were examined.

Proliferation assay. Cells were cultured in RPMI 1640 supplemented with 10% organ-conditioned medium (brain, liver, lung, or kidney) as
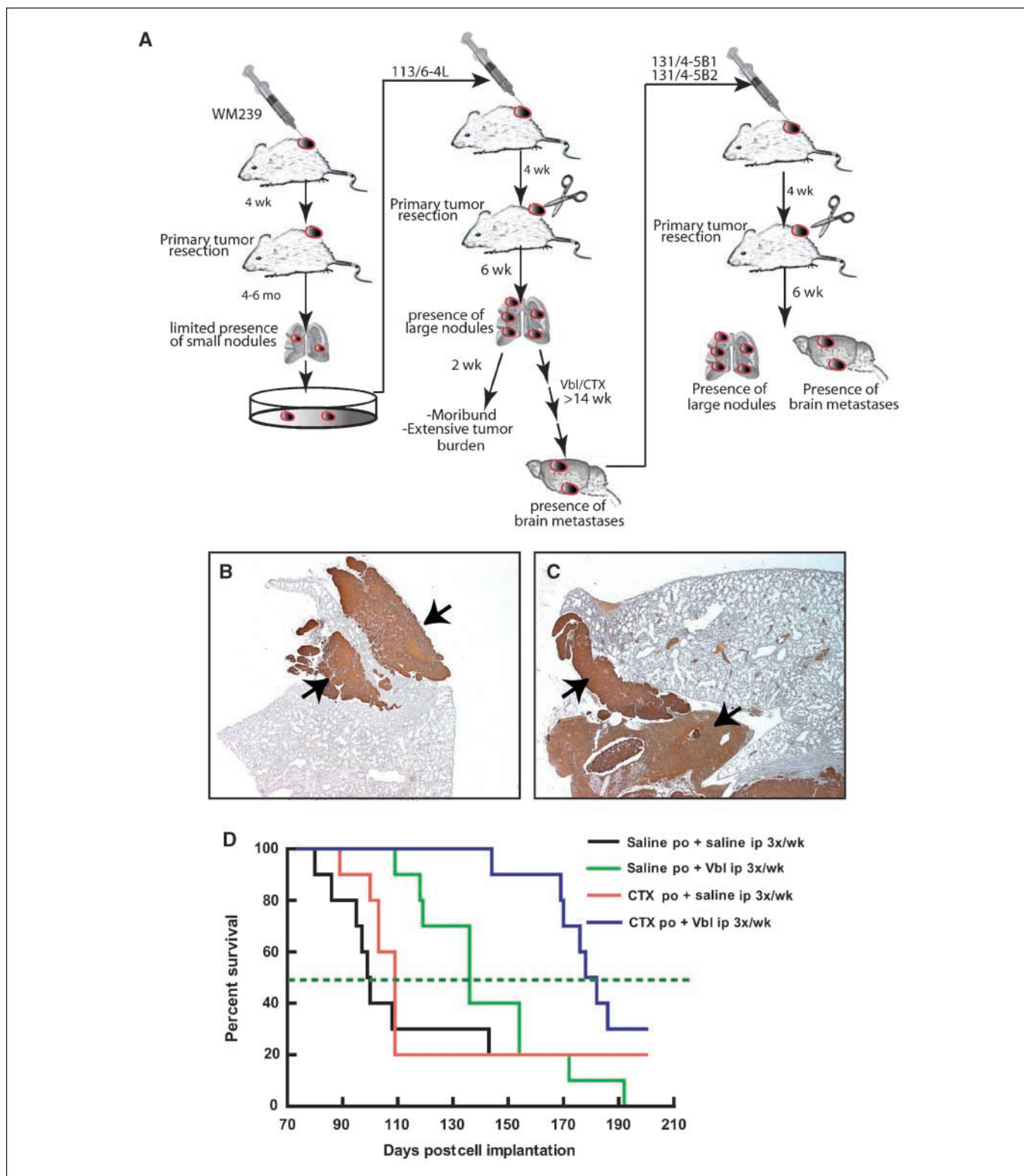
Figure 1. Schematic representation of protocol used for isolation of highly metastatic variant 113/6-4L and the effect of metronomic cyclophosphamide/vinblastine chemotherapy in the model of advanced metastatic disease. *A,* parental unselected human WM239A melanoma cells were implanted subdermally, and the primary tumors that developed were resected when they reached a size of ~400 mm³. Four to six months after resection, lungs were excised from mice and adapted for cell culture and the 113/6-4L-cell line was derived. Implantation of 113/6-4L cells resulted in high metastatic load in lungs in 6 wk postprimary tumor resection. *B* and *C,* HMB45 immunohistologic staining of lung sections, 8 wk postprimary tumor resection. *Arrows,* metastatic foci. *D,* the combination of cyclophosphamide and vinblastine used in our experiments resulted in increased survival from 99.5 to 180 d postprimary tumor resection. The rationale for the use of these drugs was based partly on evidence from clinical studies showing that a combinatorial metronomic treatment incorporating a cyclophosphamide analogue was active in patients with metastatic melanoma (19) and preclinical evidence that low-dose therapy incorporating vinblastine results in effective response in malignant melanoma patients (20). *Vbl,* vinblastine; *CTX,* cyclophosphamide.
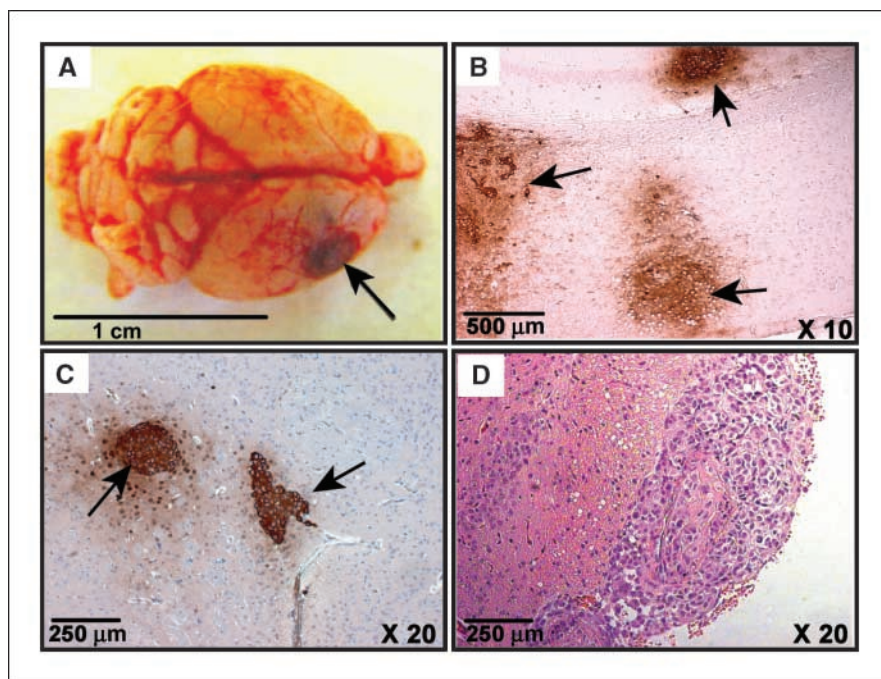
Figure 2. Orthotopic implantation of 131/4-5B1 and 131/4-5B2 cell lines results in spontaneous brain metastasis. *A*, 20% of mice that survived long-term cyclophosphamide and vinblastine therapy in the 113/6-4L model of advanced metastatic disease showed the presence of brain metastases. From these metastases, 131/4-5B1 and 131/4-5B2 cell lines were then isolated. *B* and *C*, orthotopic implantation of 131/4-5B1 and 131/4-5B2 resulted in the formation of spontaneous melanoma metastases in brain parenchyma with (*D*) occasional presence of metastatic foci in the leptomeninges (H&E staining).

described previously (8). This organ-conditioned medium contains soluble products released by the specific organ from which it was generated. The extent of proliferation was determined with the aid of MTS assay.

Adhesion assay. Melanoma cell adhesion to monolayers of murine lung (MLE) or brain (MBrE) endothelial cells was performed as described previously by Suzuki and colleagues (9). Endothelial cells were provided by Dr. J. Fidler. Monolayers of endothelial cells were seeded on 96-well plates and melanoma cell suspensions were added (5×10^5 and 1.5×10^5 cell/mL for adhesion to MLE and MBrE, respectively). Adherent melanoma cells were identified by immunostaining with HMB45 antibody as above.

Results

Development of a new human tumor xenograft model for advanced visceral (systemic) metastatic melanoma. To develop a more aggressive model of advanced metastatic melanoma, we derived a highly metastatic human melanoma cell line (named 113/6-4L) using the human cell line WM239 as a starting point (Fig. 1*A*). The method used is similar to that described previously for generating a model of advanced metastatic breast disease (10). The 113/6-4L variant was selected from diffuse spontaneous lung metastases arising from the WM239 line, which were then adapted to tissue culture. Whereas the parental line WM239 required 4 to 6 months (postprimary tumor resection) for the formation of visible metastatic nodules in lungs, the 113/6-4L variant only required 6 weeks for the formation of large macroscopic nodules in the lungs and pleural cavity. Extensive tumor burden (Fig. 1*B* and *C*) and morbidity at 8 weeks postprimary tumor resection were common. No macroscopic brain metastases were observed.

Examination of the effectiveness of combination metronomic chemotherapy on advanced metastatic melanoma. Previous work from our laboratory has shown that a combination doublet, "metronomic" chemotherapy regimen, in which each drug is chronically administered in daily, small, nontoxic doses with no prolonged breaks, was remarkably effective in the treatment of advanced, established human metastatic breast cancer in SCID mice (10). To examine whether an effective metronomic chemotherapy approach could be designed for treating advanced metastatic melanoma, we initiated concurrent combination therapy of metronomic cyclophosphamide and vinblastine using the 113/6-4L model described above, where treatment was initiated at a stage of extensive metastatic burden. This treatment regimen resulted in effective systemic disease control such that median survival was almost doubled (99.5 and 180 days for control and treated mice, respectively; Fig. 1*D*); treatment with either vinblastine or cyclophosphamide alone resulted only in reduced increases in survival (136 and 109 days, respectively). These results show that, even in an end-stage model of metastasis, the use of low-dose metronomic chemotherapy combination of vinblastine/cyclophosphamide can control systemic metastatic melanoma and highlight the importance of conducting further investigations to examine whether a vinblastine/cyclophosphamide regimen incorporating standard agents can significantly increase survival.

Development spontaneous melanoma CNS metastases. Prolonged control of systemic metastatic disease has been associated with the emergence of CNS metastases (11, 12). Thus, we examined whether the effective control of visceral melanoma metastasis mediated by vinblastine/cyclophosphamide could result in the appearance of brain metastases. Among mice that survived the long-term (170–190 days) chemotherapeutic treatment in the model noted above, 20% showed the presence of spontaneous melanotic brain metastases (Figs. 1*A* and 2*A*). We speculate that, as it has been shown to occur in human disease, this occurred as a result of altering the natural course of systemic disease in sites such as liver and lungs, thereby allowing microscopic brain metastases sufficient time to grow into larger visible lesions. From these metastases, two cell lines

Figure 3. Brain metastatic cell lines show enhanced adhesion to brain and lung endothelial cells, as well as preferential growth in brain-conditioned medium. *A,* both B1 and B2 showed a significantly higher adhesive potential to monolayers of brain endothelial cells. *B,* similarly B1 and B2, as well as 8-2L, were more efficient in adhering to monolayers of lung endothelial cells (ANOVA, *P* < 0.05). *C,* subdermal implantation of the various cell lines showed that their growth as primary orthotopic tumors did not differ. *D,* when cultured in brain-conditioned medium, only brain metastatic cell lines B1 and B2 showed an increase in proliferation when compared with the parental cell line 113/6-4L (ANOVA, *P* < 0.05).
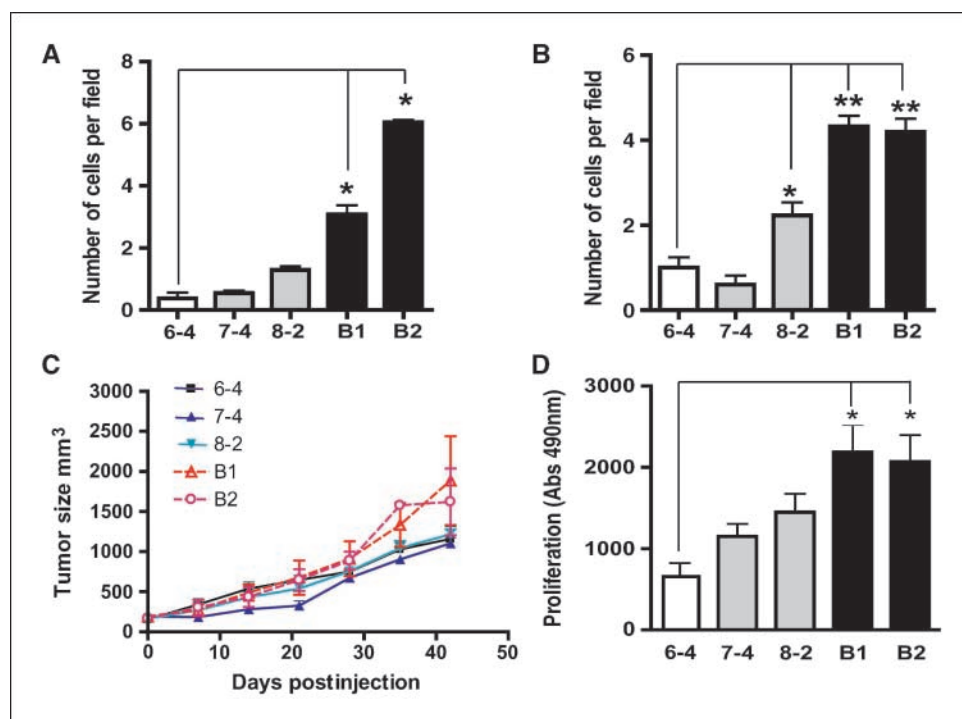
(131/4-5B1 and 131/4-5B2) were generated. These cell lines, as well as the "parental" metastatic 113/6-4L and cell lines derived from lung metastases (131/7-4L and 131/8-2L), were injected orthotopically (i.e., subdermally) in mice to examine their metastatic properties. The pattern of metastatic colonization was examined in tissues isolated from mice (days 97–180 postprimary tumor resection) that had not received any therapeutic intervention. The parental and cell lines derived from lung metastases were not able to spontaneously metastasize to the brain (Table 1). In contrast, the 131/4-5B1 and 131/4-5B2 cell lines were capable of metastasizing to the brain in approximately half (54%) of mice. Immunohistologic examination shows that multiple metastases (ranging in size of 250–500 μm in diameter) were present primarily in the brain parenchyma (Fig. 2*B* and *C*), with occasional evidence of leptomeningeal metastases (Fig. 2*D*). All cell lines metastasized with similar

efficiency to lung, liver, and kidneys, suggesting that brain-metastatic cell lines had not acquired an overall increase in metastatic potential but rather had acquired changes that specifically facilitated spread to and colonization of the brain. Both 131/4-5B1 and 131/4-5B2 constitute the first reported preclinical model of spontaneous human cancer xenograft brain metastasis, including melanoma.

Characterization of cell lines established from spontaneous brain metastases. Knowledge of the mechanisms responsible for progression of malignant tumor to brain metastases, including melanoma, is limited (13). A preferential metastatic potential of a tumor in a secondary (metastatic) site has been suggested to be mediated by acquired alterations that facilitate processes such as adhesion, or survival/proliferation in the target organ (8, 14). As such, we examined whether the ability to metastasize to brain might be influenced by alterations in adhesion of the tumor cells to

Table 1. Metastatic profiles of WM239A variants after primary tumor growth and surgical resection

Sites of metastasis	Cell lines analyzed				
	6-4L	7-4L	8-2L	4-5B1	4-5B2
Brain	0/3	0/4	0/5	3/7	4/6
Lung	3/3	2/4	4/5	4/6	4/5
Kidney	1/3	1/4	1/5	0/5	2/5
Liver	0/3	1/4	1/5	0/6	0/4

NOTE: The parental (6-4L), lung metastases–derived (7-4L and 8-2L), and brain metastases–derived (B1 and B2) cell lines were implanted orthotopically; primary tumors mice implanted orthotopically with 131/4-5B1 or 131/4-5B2 cells showed the presence of metastases. All cell lines metastasized efficiently to lung and showed minimal spread to liver and kidney.

vascular beds in the brain. In this respect, brain-metastatic cell lines 131/4-5B1 and 131/4-5B2 were found to be preferentially more efficient in adhering to murine brain and lung endothelial cells monolayers *in vitro* (Fig. 3A and B).

We next asked whether the brain metastatic phenotype might be a reflection of changes leading to increased proliferation. Examination of the growth of the various cell lines as primary tumors showed no significant difference, suggesting that the brain-metastatic cell lines have not acquired an intrinsically increased proliferative potential, at least in the dermal microenvironment (Fig. 3C). We then examined whether the brain-metastatic cell lines had acquired alterations that allowed for preferential growth in the brain microenvironment. To this end, the tumor cell lines were cultured with organ-conditioned medium. No preferential growth was observed when cell lines were cultured with liver- or kidney-conditioned medium, and only 8-2L showed a significant increase in proliferation in lung-conditioned medium (data not shown). In the case of brain-conditioned medium, only the brain-metastatic cells lines showed a significant increase in proliferation when compared with the parental cell line (Fig. 3D). Although not statistically significant, 131/7-4L and 131/8-2L cell lines seem to show a tendency of increased proliferation relative to the parental cell line 113/6-4L, which may reflect an intermediary state of increased metastatic potential that may require additional alterations to facilitate formation of metastatic disease in the CNS.

Discussion

Despite the clinical significance of CNS metastases, there is a limited understanding of the various alterations responsible for the progression of malignant tumors to a brain-metastatic phenotype, including malignant melanoma. Thus far, most of the insights about the nature of the alterations required for a brain-metastatic phenotype have been gained through the use of "experimental" metastasis models. However, these models do not reflect the complete cascade of events involved in clinical metastatic disease (15). As such, the experimental brain-metastatic cell lines generated through these models are less likely to reflect the mechanisms and genetic/epigenetic alterations that are essential for successful metastasis to CNS. A better understanding of these alterations can be gained through the use of models that recapitulate all the steps involved in metastatic cascade (i.e., spontaneous dissemination from established orthotopic primary tumors that results in the successful formation of a high rate of metastatic lesions in the brain parenchyma).

Although the spontaneous spread of melanoma to brain in rodent models has been previously documented (16), such reports are rare and only a single previous report has been described in the literature showing spontaneous and heritable metastasis to CNS. However, this model used a single clone of a murine melanoma cell line (B16), which metastasized only to leptomeninges (17) and, thus, does not recapitulate clinical disease that presents largely with metastases in the brain parenchyma (18).

The derivation of the human melanoma variants that can spontaneously and heritably metastasize in mice to the CNS from a primary tumor is unique. The procedure used to isolate these cells replicates the selection mechanisms that lead to brain metastases in malignant melanoma patients. As such, the spread of 4-5B1/B2 cell lines to CNS is likely to recapitulate the alterations that mediate this phenotype in clinical disease. This is reflected by the ability of 4-5B1/B2 to spontaneously form brain metastases in a manner that is characteristically seen in melanoma brain metastasis patients (primarily parenchymal but also with leptomeningeal localization). Our results implicate that the acquisition of a brain-metastatic phenotype is likely favored by alterations leading to increased adhesion of tumor cells to brain endothelial cells and increased proliferation in the presence of brain-derived factors. Identification of the specific molecules/interactions that may facilitate these alterations is the subject of ongoing studies. In this respect, our model is particularly advantageous in that it allows the examination of the sequence of alterations that occurs in the transition from poorly metastatic (WM239A cell line) to highly visceral metastatic variant (113/6-4L) and then to brain-metastatic phenotypes (4-5B1 and 4-5B2).

In this regard, we also note that the approaches described herein for development of a model of spontaneous CNS metastases have been recapitulated using the context of human breast cancer xenografts.[1]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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[1] Man, Francia, and Kerbel, unpublished observations.

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