The Biology of Metastasis to a Sanctuary Site

Diane Palmieri,1 Ann F. Chambers,2 Brunhilde Felding-Habermann,3 Suyun Huang,4 and Patricia S. Steeg1

Abstract

Metastasis to the brain is prevalent in solid tumors and lymphomas, and is associated with shortened survival. The brain is regarded as a sanctuary site for metastatic tumor cells where they exist partially protected from drugs by the blood-tumor barrier. Model systems for brain metastasis have been developed and are now yielding mechanistic insights into the roles of angiogenesis, energy metabolism, the Her-2 and Stat3 signaling pathways, and dormancy. Specific, new approaches to combat brain metastatic disease are needed.

Metastasis, the spread of cancer from the site of primary tumor growth to distant organs, is a leading cause of cancer morbidity and mortality. The metastatic process is complex, requiring invasion from the primary tumor, intravasation, survival, arrest and extravasation of the circulatory system and colonization of a distant site (reviewed in refs. 1, 2). In addition to these general requirements, tumor cells may acquire the ability to preferentially colonize certain organs. In theory, organ-specific metastasis may result from (a) tumor cell trapping—tumor cell arrest based on the pattern of blood flow from the primary tumor to the first capillary bed and the ability of the tumor cells to form aggregates; (b) tumor cell adhesion—specific adhesive interactions between tumor cells and the endothelia of certain organs; and/or (c) tumor cell niches—the microenvironment of a distant organ creating a permissive site for metastatic colonization. Molecular factors that may contribute to organ-specific metastasis of breast cancer to the lung and bone have been identified by experimental studies from the Massague lab, from lung- and bone-colonizing variants of a human breast carcinoma cell line (3, 4). However, we are not nearly as advanced in our understanding of central nervous system or "brain" metastases.

Brain metastases occur late in the progression of multiple types of cancer and are associated with poor patient survival. The incidence of brain metastases may be underestimated due to the presence of asymptomatic lesions, a focus on systemic lesions in a seriously ill patient, and incomplete reporting (5). Posner and Chernik reported data from 3,219 cancer patient autopsies conducted from 1970 to 1976. Intracranial, dural, and leptomeningeal lesions were found in 24%, 20%, and 8% of cases, respectively. Intracranial metastases were most prevalent from lung cancer (3.1%), followed by breast cancer (2.9%), melanoma (2.8%), and lymphoma (1.7%); another 4.2% of brain metastases came from primary tumors of unknown histology (6, 7).

Recent studies investigating patients with metastatic lung and breast cancer reported alarming rates of brain metastases: among metastatic breast cancer patients with Her-2+ tumors, the incidence of brain metastases varied from 26% to 48% (8–12). Where examined, the majority of patients were responding to trastuzumab treatment or had systemically stable disease when they developed brain metastases. Omuro et al. (13) followed patients with non–small cell lung carcinoma treated with, and responding to, the epidermal growth factor receptor kinase inhibitor gefitinib. The 5-year incidence of brain metastasis was 60%; 33% of responding patients developed brain metastases as the first site of relapse.

These studies suggest that the incidence of brain metastases may be increasing. The causes of an increased incidence of brain metastases are unknown but theories have been posited. As patients live longer due to the use of new molecular therapies, additional brain metastases likely occur. The brain may represent a preferential site of metastasis because many of the new therapies (and virtually all of the traditional chemotherapeutics) cannot cross the blood-brain barrier or what is left of the blood-brain barrier once a tumor forms, the blood-tumor barrier. Thus, relapses occur in a “sanctuary” site. On the other hand, the incidence of brain metastases may not have risen, but the increased use of refined imaging and more attention to neurologic symptoms may facilitate their detection.

Experimental model systems can help to sort out tumor and host factors that contribute to the intrinsic brain tropism of cancer cells versus factors that are consequences of detection or therapeutic advances. This review will summarize progress in the development and validation of model systems for brain metastases of cancer. Recent advances in defining the molecular
underpinnings of brain tropism will be discussed, and future needs enumerated. Because brain metastases represent an unmet medical need, it is hoped that information from these models can be rapidly translated to the clinic.

**Experimental Model Systems of Brain Metastasis**

Rodent model systems have been reported for brain metastases in melanoma (reviewed in ref. 14), lung carcinoma (15, 16), and breast carcinoma (17–24). A model of metastasis to the choroid of the eye was reported in which tumor cells also invaded the brain (25). Table 1 lists the salient characteristics of several prominent models; important facets of the BCM2 breast cancer brain metastasis model are presented in Fig. 1.

Metastatic murine or human cell lines are often the starting point in the development of these models. In contrast, the BCM2 brain line was established from circulating tumor cells from a patient with stage IV breast cancer (Fig. 1). To isolate a brain tropic line, cell lines were subjected to multiple sequential rounds of in vivo selection in mouse models by harvesting the initial brain lesions that developed after injection. These tumor cells were established as ex vivo cultures, re-injected into a new host, and again harvested from resulting brain metastases. The characteristics of several lines have been reported after sequential rounds of selection, including a progressively higher incidence of brain metastases, fewer metastases to other organs, and/or shortened host survival. Routes of injection included the tail vein, the left ventricle of the heart, or the internal carotid artery. The latter routes present technical challenges but favor brain colonization because the cerebral microcirculation is the first capillary bed encountered. Another in vivo model with some relevance to brain metastasis is the stereotactic implantation of tumor cells into the brain, although this procedure eliminates many steps of the metastatic process and causes trauma to the brain that may affect tumor cell growth. In vitro models include cocultures of tumor cells and brain-derived cells (26, 27), transendothelial cell invasion using cultured brain microvascular endothelial cells (28–31), and ex vivo brain slice invasion assays (32).

Quantification of brain metastases is typically done histologically, in which serial sections of the brain are examined. Standardization is needed so that preclinical efficacy experiments can be easily interpreted. In some experiments, the incidence of brain metastases is noted. However, given the technical difficulty of intracardiac or intracarotid artery injection in the mouse model, it is sometimes unclear if an animal without tumor signals in the brain received the full dose.

<table>
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<tr>
<th>Tumor cells</th>
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<th>Incidence and prevalence of brain metastases</th>
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<td>Melanoma</td>
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<td>B16F1</td>
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<td>B16-B7b</td>
<td>i.c.</td>
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<td>B16-B7n</td>
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<td>B16-B10n</td>
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<td>Brain, tropism lost with in vitro culture</td>
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<td>B16-B14b</td>
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<td>Brain and lung metastases, prominent host inflammation</td>
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<td>B16-B15b</td>
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<td>B16-F10</td>
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<td>B16-F10-B2</td>
<td>i.v.,* i.c.</td>
<td>Lung metastases, dorsal cerebral metastases</td>
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<td>(14)</td>
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<td>K-1735 lines</td>
<td>i.a.</td>
<td>Brain parenchyma</td>
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<td>MeWo 70W (H)</td>
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<td>Mel57-EGFP (H)</td>
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<td>Brain parenchyma</td>
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<td>A375Br (H)</td>
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<td>5 of 5 mice positive</td>
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<td>(52)</td>
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<td>TXM-18 (H)</td>
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<td>5 of 5 mice positive</td>
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<td>Lung cancer (NSCLC)</td>
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<td>A549(H)</td>
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<td>Grafted onto lungs</td>
<td>2 of 3 mice positive</td>
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<td>Breast cancer</td>
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<td>i.c.</td>
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<td>Maestro¹</td>
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<td>BR3</td>
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<td>3 of 10 positive</td>
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<td>MDA-MB-43S Br1 (H)</td>
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<td>BCM2 brain G2 circulating tumor cells</td>
<td>i.v.</td>
<td>19 of 23 brain plus some lung and bone metastases</td>
<td>Noninvasive bioluminescence</td>
<td>(24)</td>
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<td>F-luc</td>
<td>i.v.</td>
<td>19 of 23 brain plus some lung and bone metastases</td>
<td>Noninvasive bioluminescence</td>
<td>(24)</td>
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<td></td>
<td>i.a.</td>
<td>10 of 10 brain</td>
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Abbreviations: i.c., intracardiac; i.a., intracarotid artery; (H), human tumor cell line.
¹Treated with cytochalasin B and colchicine prior to i.v. injection.
¹Using dissected brains.
of tumor cells and failed to develop brain metastases, or if the injection “missed.” The use of luciferase-tagged tumor cells and noninvasive bioluminescence imaging of the animals immediately after injection and at later time points can help to clarify this issue.

For quantification as well as qualitative analysis of brain metastatic burden, histological examination is important because it not only reveals the extent of metastatic growth but also the size and shape of metastatic brain lesions. The number of brain metastases in step sections is therefore a reliable indicator, but questions still arise: does one score micrometastases, consisting of only a few cells, the same way as large “cannonball” metastases? Because patients are diagnosed with large metastases, and we do not fully understand the implications of micrometastases, it may be judicious to tally the two types of lesions separately. And what size cutoff determines micrometastasis? These models are aided by coupling with improved imaging techniques such as bioluminescence or fluorescence imaging of luciferase or fluorescent protein–labeled tumor cells, respectively. Additionally, superparamagnetic or micron-sized iron oxide particles, which are detected in magnetic resonance imaging scans, may provide insights (33–35). In a study of enhanced green fluorescent protein–labeled human breast carcinoma cells, imaging of dissected brains gave only semiquantitative results because a fluorescent signal could represent a solitary large brain metastasis or a collection of micrometastases upon histologic analysis (36). These data underscore the need for a complete histologic examination.

Although the establishment of a handful of model systems represents a significant accomplishment, additional models are needed to represent the heterogeneity of brain metastasis observed in the clinic. Almost no comparison has been made between the molecular characteristics of model systems and those of clinical brain metastasis specimens.

The Complex Roles of Angiogenesis and Permeability

Angiogenesis is an important aspect of tumor metastasis that has been reported in several brain metastasis models. Multiple facets of the vascular system have been investigated, including angiogenesis, the formation of new blood vessels, the utilization (or co-option) of existing blood vessels, and the permeability of the vascular system. The conclusions of the reported studies tend to vary by model but may describe the heterogeneity found in human disease.

The Fidler laboratory examined several aspects of angiogenesis in model systems including the brain. The brain is a highly vascularized organ. Capillary densities in brain metastases were actually lower than those of the surrounding brain (ref. 37; reviewed in ref. 38). An assay of vessel permeability was
reported in which sodium fluorescein was injected into mice with experimental brain metastases of human KM12C tumor cells. For micrometastases smaller than 0.1 mm², the blood-tumor barrier was intact; however, the blood-tumor barrier leaked in larger (>0.4 mm²) metastases but still excluded therapeutic drugs (38).

Recent studies have examined the roles of vascular endothelial growth factor (VEGF), which influences both angiogenesis and blood vessel permeability (reviewed in refs. 39, 40). The 121 amino acid splice form of VEGF is soluble, whereas the other 145, 165, and 189 amino acid splice forms are extracellular matrix-bound, but are released and activated in the presence of proteases (41). Brain-colonizing sublines of the human MDA-MB-231 breast carcinoma cell line produced greater amounts of VEGF and another angiogenic factor, IL-8, in vitro than the parental cell line. The VEGF receptor inhibitor, PTK787, reduced the tumor burden, capillary density, and mitotic rate of brain metastases resulting from carotid artery injection of tumor cells in vivo (42). Similarly, antisense VEGF-165 transfectants of PC14PE6 lung adenocarcinoma and KM12SM colon carcinoma cells exhibited a decreased incidence of brain metastases following intracarotid injection. Halofuginone, a type I collagen inhibitor, reduced the size and vascular density of melanoma cells intracranially implanted into the rat brain (43).

Different outcomes were observed in other model systems, which may affect the efficacy of antiangiogenic therapies in this site. Transfection of VEGF-121 or -165 into the H226 human lung squamous carcinoma cell line exerted no effects on angiogenesis or brain metastasis following intracarotid injection (44). Mel57 human melanoma cells produced little endogenous VEGF but established infiltrative brain metastases in mice by co-opting existing peritumoral vessels (45), indicating that the preexisting vasculature can contribute to metastatic growth. Interestingly, the blood-brain barrier remained intact in these co-opted vessels. When the VEGF-121, -165, and -189 splice forms were transfected into Mel57 cells side-by-side, the three isoform transfectants induced comparable endothelial cell proliferation in vitro. The VEGF-121 transfectants failed to initiate angiogenesis but induced the dilation and permeability of existing peritumoral vessels in vivo. In contrast, VEGF-165 transfectants initiated angiogenesis, disrupted the blood-brain barrier, increased permeability, and produced the most marked metastatic growth by imaging (46). These findings indicate that different VEGF forms can serve different functions in the context of the brain. Analysis of VEGF expression and vascularization patterns in a small cohort of human clinical brain metastases suggested that conclusions from the model systems were applicable to human metastases (47). Likewise, the effect of antiangiogenic agents varied in the brain. Treatment of the VEGF-165 transfectants with the maximum tolerated doses of the VEGF receptor inhibitor ZD6474 resulted in a diminution of the VEGF-165 phenotype including restoration of the blood-brain barrier and a reduction in the size of metastases on contrast-enhanced magnetic resonance imaging (48). However, histologic analyses showed continued metastatic progression via co-option of existing vessels (37, 48). The co-option process was thought to be sufficient to sustain tumor viability as no hypoxia or necrosis was observed in this study. In contrast, treatment with a lower dose of inhibitor reduced new vessel formation but left the blood-tumor barrier permeable. The authors propose that lower dose “antiangiogenic” therapy may facilitate the delivery of other chemotherapeutics with a clinical benefit, despite the lack of a classical antiangiogenic effect (48).

In conclusion, factors including concentrations of angiogenic factors, the mix of angiogenesis factors (such as splice forms of VEGF), the level of antiangiogenic drug, and the availability of an existing vessel network for co-option may all contribute to antiangiogenesis drug responsiveness in brain metastases. Because bevacizumab is a large monoclonal antibody, its effectiveness in the brain is expected to be poor. Studies of clinically relevant VEGF receptor tyrosine kinase inhibitors and other small molecule angiogenesis inhibitors, conducted at doses that are achievable in humans, will be important for further understanding this complex situation. These studies can not only address angiogenesis but the effects of altered blood vessel permeability on the delivery of other drugs to brain metastases.

Mechanistic Insights

Her-2

The Her-2 receptor tyrosine kinase belongs to one of the most studied signal transduction pathways in cancer. Amplification or overexpression of Her-2 is associated with poor patient survival in breast cancer cohort studies. Transfection data, genetically engineered mice, and the use of inhibitors such as trastuzumab (herceptin) revealed effects on tumor cell proliferation, colonization, and motility in vitro, and tumorigenicity and metastasis to the lung and bone in vivo (reviewed in ref. 49). Although trastuzumab has shown clinical activity in the metastatic and adjuvant settings in combination with chemotherapy, one of the greatest challenges for patients with Her-2 positive tumors is the development of brain metastases (8–10, 12, 50). The association of Her-2 overexpression and brain metastases may be caused by at least three factors: (a) the inability of an antibody such as trastuzumab to penetrate the blood-tumor barrier, (b) the longer life span afforded patients receiving therapy, and/or (c) the possibility that Her-2 changes the natural history of breast cancer to increase its brain metastatic potential. A brain tropic subline of the human MDA-MB-231 breast cancer cell line (231BR) was transfected with low (4- to 8-fold) or high (22- to 28-fold) amounts of Her-2 to test the latter possibility. Intracardiac injection of the control and Her-2–transfected 231BR cells produced similar numbers of brain micrometastases, but the Her-2 transfectants produced 2.5- to 3-fold greater numbers of large (>50 microns²) brain metastases (36). The data provide the first evidence that Her-2 overexpression changes the natural history of breast cancer to promote outgrowth of tumor cells in the brain. These data reinforce the need to develop brain-permeable Her-2 inhibitors. Laptatinib, a small molecule inhibitor of Her-2/epidermal growth factor receptor, is undergoing clinical testing for brain metastases of breast cancer.
Stat3

Stat3 has been implicated in tumor cell apoptosis and angiogenesis, presumably through its transcriptional regulatory activity. Real et al. (51) reported that epidermal growth factor–induced Stat3 expression in a brain metastatic variant of the MDA-MB-435 breast carcinoma cell line. Overexpression of Stat3 led to elevated expression of the antiapoptotic Bcl-2 protein, suggesting a survival and resistance advantage. The Huang lab reported that Stat3 overexpression promoted the brain metastasis of the A375 human melanoma cell line, confirming the role of Stat3 in the promotion of brain metastasis (52). Genes in which expression was altered by increased Stat3 expression included several involved in invasion and angiogenesis, including matrix metalloproteinase-2, basic fibroblast growth factor, and VEGF, suggesting that Stat3 may serve multiple prometastatic roles. It will be of interest to determine the efficacy of Stat3 inhibitors in this model system.

Energy metabolism

The Felding lab isolated circulating tumor cells from a metastatic breast cancer patient and, using several rounds of in vivo selection in mice, established cell lines that metastasized to the mouse brain or bone (Fig. 1). Comparing proteomic expression profiles of the parental cells against their brain or bone tropic variants, the brain-metastasizing tumor cells overexpressed enzymes involved in aerobic glycolysis and the TCA cycle. These enzymes included triose phosphate isomerase, phosphoenolpyruvate carboxylase, aconitase hydratase, isocitric dehydrogenase, and mitochondrial malate dehydrogenase. This coordinated up-regulation of proteins suggests a propensity for energy procurement from glucose oxidation. In addition, AMP kinase, glutathione, and proteins involved in fatty acid oxidation and the pentose phosphate pathway were specifically up-regulated (24). The results indicate that breast cancer cells capable of colonizing the brain either adapt to the energy metabolism in the brain or they may be predestined to thrive under those conditions. Consistent with these data, the brain metastatic cell line was relatively insensitive to glucose deprivation and to glutathione-dependent oxidative stress induced by an inhibitor of the ubiquitin-proteasome system in vitro (24). These findings indicate that unique energy management of metastatic tumor cells in the brain may provide insight into mechanisms that allow metastatic cells to survive and proliferate in the brain microenvironment, and shed light on the drug responsiveness of tumor cells that successfully colonize the brain.

Other mechanistic insights

The use of brain metastasis models has provided several additional mechanistic insights. A role for plasmin in melanoma intravasation into and subsequent colonization of the brain was reported (53). Proteins known to be associated with metastatic tumor cell activity have also been functionally implicated in brain metastasis, including multiple proteases (54–58), Bcl family members (59), and integrin α3β1 (60). In addition, the neurotrophins constitute a class of mediators which regulate tumor cell protease production. Normally involved in neuronal cell survival, differentiation, and death, neurotrophins can be produced by astrocytes or overexpressed by brain metastatic melanoma cells, leading to heparanase production (reviewed in ref. 61). Other neurotransmitters have been reported to be chemotactic for tumor cells, suggesting an interaction of the tumor and the brain microenvironment (55).

Molecular Characterization of Brain Metastases

In addition to the use of model systems, human brain metastatic tissue is available in limited quantities from craniotomies and autopsies, and several molecular analyses have been reported. At a gross level, gains in chromosomes 15q and 20q were identified in brain metastases of prostate cancer (62). Increased p21 expression was reported in breast cancer brain metastases as compared with primary tumors (63). Although p21 has a classical role as a cell cycle inhibitor, it is also correlated with innate and chemotherapeutic-induced resistance by an undefined mechanism. Reduced expression of the metastasis suppressor genes Kiss1, Kait, Brms1, and Mkb4 was reported in breast cancer brain metastases as compared with unlinked primary tumors (64). These data constitute ideal leads for testing in model systems.

Alterations in the DNA methylation of several genes were reported in brain metastases, including p16, DAP-kinase, thrombospondin-1, retinoblastoma 1, O6-methylguanine DNA methyltransferase, glutathione-S-transferase P1, p14, cyclin D2, retinoic acid receptor B, twist, and Hin-1 (65, 66). It is not known whether these findings are specific to the brain as opposed to other sites of metastasis, but suggest the hypothesis that drugs regulating gene expression such as HDAC inhibitors or DNA methyltransferase inhibitors may have efficacy in the brain.

The Issue of Dormancy in Brain Metastases

Micrometastases are rarely discussed in the literature regarding human brain metastasis. From a clinical standpoint, patients are diagnosed when brain metastases are sufficiently large to be observed with imaging, containing millions of tumor cells. However, these large metastases emanated from a micrometastasis, and the processes controlling their outgrowth are germane to metastasis prevention studies. The elimination of occult micrometastases serves as the rationale behind giving whole brain radiation therapy. Recently, Baumert et al. (67) reported autopsy results from 45 patients with brain metastases who underwent gamma knife radiosurgery. In 48 of 76 metastases, infiltration of tumor cells in small nests, i.e., micrometastases, was apparent in the area surrounding the border of the larger brain metastases. These lesions seem similar to those seen in the MDA-MB-231BR animal model.5

Another important question is whether all micrometastases are progressively growing, or alternatively, whether some enter periods of tumor dormancy. Certain cancers such as those of the breast and prostate recur years after the primary diagnosis in some patients, suggesting that the tumor cells can lie dormant

5 P.S. Steeg and D. Palmieri, unpublished data.
in a distant location for long periods of time (68). The forces that induce dormancy or reawaken these tumor cells are largely unknown, but their identification could lead to new forms of therapy.

The Chambers laboratory labeled 231BR human breast carcinoma cells with micron-sized iron oxide particles and followed their fate in vivo using magnetic resonance imaging (33). If the tumor cells successively divided, the particles were apportioned between daughter cells and an imaging signal was lost. Single tumor cell “voids” were detectable 24 h after intracardiac injection and the fate of these cells was followed by repeated magnetic resonance imaging scanning for 1 month. Approximately 94% of tumor cells initially seeding the brain were not detectable by the end point of the experiment. Another 1.6% of tumor cells proliferated to form macroscopic metastases, detectable by a transsected enhanced green fluorescent protein label, whereas 4.5% of tumor cells were apparently dormant, retaining the micron-sized iron oxide particle labels for the duration of the experiment (33). These data provide the first in vivo evidence for dormancy in a model of brain metastasis of breast cancer. Approximately a 3:1 ratio of dormant to colonizing tumor cells indicates that a potent subpopulation of cells exists that should be the focus of molecular and preclinical research to generate information aimed at successfully targeting those potentially relapse-initiating cells.

Conclusions and Future Perspectives

The development of model systems for brain metastasis represents an important advance in this field. Additional model systems representing each cancer histologic type are needed so that the heterogeneity inherent in the patient population can be modeled. The current models have limitations, many of which can be addressed by further experimentation. These include a lack of validation against clinical brain metastases from carcinotomies or autopsies, to ensure that the pathways active in the model are present in the human situation. Another potential weakness is the failure of models to incorporate the standard treatment, radiation therapy. Ideally, animals could receive a form of radiotherapy such as a dose approximating gamma knife radiosurgery, so that responses in this setting could be determined. Improvements are needed in the quantification of brain metastases in model systems. Imaging, although promising, fails to distinguish a collection of micrometastases from larger metastases, leaving us with tedious microscopic examinations as end points. Finally, almost nothing has been reported on cognitive function in animal models of brain metastases. Cognitive function is severely affected by brain metastases and the complications of their treatment. One could envision maze, agility, and learning tests, now applied in other fields, being incorporated as end points.

It is remarkable that, given our abilities in mouse models, a comprehensive data set has not been published of drug permeability into the normal brain, brain tissue around metastases (which may have altered permeability), large metastases, and micrometastases. Steadily, brain permeability is given increasing consideration in drug development. Given the fact that brain metastases represents an unmet medical need, it would make sense to prioritize the testing of new therapeutics in a brain metastasis model.

New strategies for the delivery of drugs to brain metastases are also a high priority. Strategies such as neural stem cell homing (69, 70), immune approaches (71), and novel delivery vehicles (72) are emerging and represent high-risk, high-impact avenues of exploration.

References

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