Mol. Cells *30*, 93-98, August 31, 2010
DOI/10.1007/s10059-010-0133-9

Minireview

Molecules and Cells

The Brain Microenvironment and Cancer Metastasis

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The process of metastasis consists of a series of sequential, selective steps that few cells can complete. The outcome of cancer metastasis depends on multiple interactions between metastatic cells and homeostatic mechanisms that are unique to one or another organ microenvironment. The specific organ microenvironment determines the extent of cancer cell proliferation, angiogenesis, invasion and survival. Many lung cancer, breast cancer, and melanoma patients develop fatal brain metastases that do not respond to therapy. The blood-brain barrier is intact in and around brain metastases that are smaller than 0.25 mm in diameter. Although the blood-brain barrier is leaky in larger metastases, the lesions are resistant to many chemotherapeutic drugs. Activated astrocytes surround and infiltrate brain metastases. The physiological role of astrocytes is to protect against neurotoxicity. Our current data demonstrate that activated astrocytes also protect tumor cells against chemotherapeutic drugs.

INTRODUCTION

The major cause of death from cancer is metastases that are resistant to conventional therapy. Metastases can be located in different organs or in different regions of the same organ, and the anatomic location of metastases plays a critical role in determining response to therapy. Primary tumors in general and metastatic lesions in particular are biologically heterogeneous and contain multiple cell populations with diverse characteristics of growth rate, karyotype, cell surface receptors, antigenicity, immunogenicity, enzyme profile, hormone receptor composition, sensitivity to different cytotoxic drugs, production of extracellular matrix proteins, adhesion molecule profile, angiogenic potential, invasiveness, and metastatic potential (reviewed in Talmadge and Fidler, 2010).

The outcome of the metastatic process depends on multiple and complex interactions of metastatic cells with host homeostatic mechanisms (Fidler, 2003; Langley and Fidler, 2007). Clinical observations of cancer patients and studies with experimental rodent tumors have concluded that certain tumors produce metastasis to specific organs independent of vascular anatomy, rate of blood flow, and number of tumor cells delivered to each organ. Indeed, the distribution and fate of hematogenously disseminated, radiolabeled melanoma cells in experimental animals conclusively demonstrated that tumor cells can reach the microvasculature of many organs, but growth in the organ parenchyma occurred in only specific organs (Fidler and Talmadge, 1986; Weiss, 1985).

More than a century ago, the English pathologist, Stephen Paget, questioned whether the organ distribution of metastases produced by different human neoplasms was due to chance and analyzed more than 900 autopsy records of women with breast cancer. His research concluded that the nonrandom pattern of metastasis was not due to chance but, rather, that certain tumor cells (the "seed") had a specific affinity for the milieu of certain organs (the "soil"). Metastases resulted only when the seed and soil were compatible (Paget, 1889).

Some 40 years later, J. Ewing challenged Paget's seed and soil theory and proposed that metastatic dissemination occurred by purely mechanical factors that are a result of the anatomical structure of the vascular system (Ewing, 1928). Clinical observations of cancer patients and studies with experimental rodent tumors have revealed that certain tumors produce metastasis to specific organs independent of vascular anatomy, rate of blood flow, and number of tumor cells delivered to each organ. Experimental data supporting the "seed and soil" hypothesis of Paget were derived from studies on the preferential invasion and growth of B16 melanoma metastases in specific organs. The mouse melanoma cells were injected into the circulation of syngeneic mice. Tumor growths developed in the lungs and in fragments of pulmonary or ovarian tissue implanted intra-muscularly. In contrast, metastatic lesions did not develop in renal tissue implanted as a control or at the site of surgical trauma. This study confirmed that sites of metastasis are determined not solely by the characteristics of the neoplastic cells but also by the microenvironment of the host tissue (Hart et al., 1981).

Ethical considerations rule out the experimental analysis of cancer metastasis in patients, but the introduction of peritoneovenous shunts for the palliation of ascites in women with progressive ovarian cancer has provided the opportunity to study some of the factors that affect metastatic spread in humans. Good palliation with minimal complications was reported for 29 patients with ovarian cancer. The autopsy findings in 15 patients substantiated the clinical observations that the shunts do not significantly increase the risk of metastasis. In fact, despite

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Received August 16, 2010; accepted August 19, 2010; published online August 19, 2010

Keywords: astrocytes, brain metastasis, organ microenvironment, seed and soil hypothesis

continuous entry of millions of tumor cells into the circulation, metastases in the lung (the first capillary bed encountered) were rare (Tarin et al., 1984).

A current definition of the "seed and soil" hypothesis consists of three principles. First, neoplasms are biologically heterogeneous and contain subpopulations of cells with different biologic properties (Fidler, 1990; Fidler and Kripke, 1977; Hart et al., 1981; Talmadge and Fidler, 1982). Second, the process of metastasis is highly selective for cells that can complete all of the steps in the pathogenesis of metastasis (Fidler, 1973; Fidler and Talmadge, 1986). Although some of the steps in this process contain stochastic elements, as a whole, metastasis favors the survival and growth of a few subpopulations of cells that preexist within the parent neoplasm (Fidler and Kripke, 1977; Talmadge and Fidler, 1982). Thus, metastases can have a clonal origin, and different metastases can originate from the proliferation of different single cells (Hu et al., 1987; Talmadge et al., 1982). Third, the outcome of metastasis depends on multiple interactions of metastatic cells with homeostatic mechanisms that include the organ microenvironment which tumor cells exploit for their own gain (Fidler, 2003; Langley and Fidler, 2007).

Brain metastasis

Of tumors found in the brain, more than 30% are metastatic lesions produced by cancers such as lung, breast, melanoma, colon, and renal (Landis et al., 1998; Norden et al., 2005; Sawaya et al., 2001). The progressive growth of metastases in the brain is often associated with the terminal stage of the disease. The treatment of choice for a solitary metastasis is surgical excision plus radiation (Sawaya et al., 2001) while, for multiple metastases in the brain and meningeal carcinomatosis, radiation and/or chemotherapy are employed. Although brain metastases can be surgically removed without producing severe neurological complications, the prognosis is poor. After surgery alone, the median survival time ranges from 4 to 6 months; with surgery and radiation, the median survival time may exceed 6 months. A major reason for these poor results is the recurrent growth of tumors at the site of resected lesions, as well as the development of multiple metastases in other areas of the brain (Sawaya et al., 2001). Improvements in treatment of brain metastasis can only come from a better understanding of the biology of these lesions.

***In vivo* models to study the biology of brain metastasis**

To better understand the biology of cancer metastasis to the brain, we developed a murine model in which tumor lesions in the brain are produced by the injection of metastatic cells into the internal carotid artery of anesthetized mice. In this model, the high incidence of brain lesions and the low incidence of visceral lesions allow for studies of pathogenesis of brain tumors and especially brain metastases.

We developed a specialized injection technique by introducing cells into the external or internal carotid artery (Fujimaki et al., 1993; Schackert and Fidler, 1988a). Mice are anesthetized by i.p. injection of pentobarbitol sodium, restrained on a cork board on the back, and placed under a dissecting microscope. The carotid artery is prepared for an injection distal to the point of division into the internal and external carotid arteries. A ligature of 5-0 silk suture is placed in the distal part of the common carotid artery. A second ligature is placed and tied loosely proximal to the injection site. A sterile cotton tip applicator was inserted under the artery just distal to the injection site to elevate the carotid artery. This procedure controls bleeding from the carotid artery by regurgitation from distal vessels. The artery is nicked with a pair of microscissors, and a < 30-gauge glass cannula is inserted into the lumen. To assure proper delivery, the tumor cells are injected slowly, and the cannula is removed. The second ligature is tightened, and the skin is closed by sutures. Since the injection of cells into the carotid artery of nude mice simulates the hematogenous spread of tumor emboli to the brain, we use this technique to examine the last steps of the metastatic process: release of tumor cells into the circulation, arrest in capillaries, penetration and extravasation into the brain through the blood-grain barrier, and continuous growth of cells in the brain tissue (Poste and Fidler, 1980).

The origin of brain metastases

Clinical observations have suggested that brain metastases produced by many solid tumors occur late in the disease (Tsukuda et al., 1983) and have raised the question of whether brain metastases are produced by cells populating lymph node or visceral metastases, *i.e.*, metastasis of metastases. Indeed, it has been proposed that metastasis by solid tumors occurs by the initial spread of cells to a generalizing site, such as regional lymph nodes, where malignant cells proliferate and then spread to additional organs. This process has been termed the "metastatic cascade" (Viadana et al., 1978; Weiss, 1985). One consequence of this process is that metastasis can occur after a primary lesion has been surgically removed because the generalizing site remained intact (Kanematsu et al., 1988). For the pathogenesis of brain metastasis, this is not an academic issue. If brain metastasis occurs by the metastasis of metastases, then aggressive, prophylactic resection of lymph node or visceral metastases may reduce the risk of development of fatal brain lesions. On the other hand, if brain metastasis occurs by the direct spread of specialized metastatic cells from the primary lesion (Nicolson, 1988), then prophylactic dissection of extracranial metastases may not prevent brain metastasis from occurring. Thus, it is important for neurooncologists to determine whether or not brain metastasis represents shedding from generalizing site metastases or the primary tumor. One surrogate for that determination is the metastatic potential of the cells.

We studied the correlation between the formation of brain metastasis and the malignant growth potential of seven human melanoma cell lines, isolated from lymph node metastases or from brain metastases, and the potential of three variants of the mouse K-1735 melanoma (Zhang et al., 1991). Growth rates in different concentrations of fetal bovine serum and colony-forming efficiency in semisolid agarose were measured, and the tumorigenicity and metastatic ability were determined in nude mice (for the human melanoma cell lines) or in syngeneic mice (for the K-1735 variants). The ability to form brain metastasis was tested by injection of cells into the carotid artery. A high colony-forming efficiency in agarose, especially at concentrations of agarose greater than 0.6% (Li et al., 1989) corresponded with high tumor take rates, rapid tumor growth rates, and metastatic colonization of the lungs of the recipient mice (Zhang et al., 1991). For the human melanomas, the lymph node metastasis-derived cells were more tumorigenic and metastatic than the brain metastasis-derived cells. In the K-1735 mouse melanoma, the tumorigenic and metastatic behavior of the cells after i.v. and s.c. injection corresponded with growth in agarose cultures (Li et al., 1989; Zhang et al., 1991). However, for growth in the brain after intracarotid injection, the different melanoma cell lines showed similar frequencies of tumor take, regardless of tumorigenicity in other sites of the recipient mice. The results from the human and mouse melanoma cell lines show that the brain metastasis-derived cell lines were not more malignant than the lymph node or lung metastasis-derived cells.

Thus, the data imply that the production of brain metastasis is not always the final stage of a metastatic cascade (Zhang et al., 1991).

Unique patterns of brain metastasis produced by different human carcinomas

Human tumor cells from carcinomas of the colon, breast, kidney, and lung were injected into athymic mice either by a direct intracerebral route or into the internal carotid artery. All carcinoma cells invaded through the blood-brain barrier and produced progressively growing lesions in the brain parenchyma. Unique patterns of growth were discernible among the carcinomas. Subsequent to direct i.c. injection, all the human carcinoma cell lines grew in the brain of nude mice, thus demonstrating that if carcinoma cells can reach the brain parenchyma, they can proliferate there. There was more tumor growth in the parenchyma than in other regions of the brain. The seeding of tumor cells in the brain parenchyma reveals that some cells from all of the carcinoma cell lines tested were able to cross the blood-brain barrier (Gay et al., 1987; Groothuis et al., 1982).

Discrete colony formation by the colon carcinomas was seen in both cell lines injected. Notable is the growth by extension via the corpus callosum to the left hemisphere subsequent to injection of tumor cells into the right hemisphere. This result demonstrates a possible mechanism of secondary metastasis within the brain, leading to undetected satellite micrometastases at the time of diagnosis and surgery that could give rise to early recurrences of tumors close to the surgical cavity (Schackert et al., 1988).

Neoplastic angiogenesis

To produce metastasis, tumor cells must complete a series of sequential and selective steps (Fidler, 2003; Langley and Fidler, 2007). Failure to complete even one step eliminates the cells from the process (Aukerman et al., 1986). As discussed previously, to produce brain metastasis, tumor cells must reach the vasculature of the brain, attach to the microvessel endothelial cells, extravasate into the parenchyma, proliferate (by responding to growth factors), and induce the formation of new vasculature (Fujimaki et al., 1993; Schackert and Fidler, 1988b).

The growth and spread of neoplasms is dependent on the establishment of adequate blood supply, *i.e.,* angiogenesis (Carmeliet and Jain, 2000; Folkman, 1995). The onset of angiogenesis is determined by the balance between proangiogenic and antiangiogenic molecules at the local tissue level (Folkman, 1995; Liotta et al., 1991). Angiogenesis can occur by either sprouting or nonsprouting processes (Risau, 1997). Sprouting angiogenesis occurs by branching (true sprouting) of new capillaries from preexisting vessels. Nonsprouting angiogenesis results from the enlargement, splitting, and fusion of preexisting vessels produced by the proliferation of endothelial cells within the wall of a vessel. Transcapillary pillars (or transluminal bridges) are sometimes observed in enlarged vessels produced by nonsprouting angiogenesis (Risau, 1997). This type of angiogenesis can concurrently occur with sprouting angiogenesis in the vascularization of organs or tissues such as the lung, and heart (Carmeliet et al., 1996). The mechanism of nonsprouting angiogenesis in metastasis regulated by vascular endothelial growth factor (VEGF), also called vascular permeability factor (VPF) (Ferrara and Henzel, 1989; Senger et al., 1983). VEGF stimulates the proliferation and migration of endothelial cells and induces the expression of metalloproteinases and plasminogen activity by these cells (Ferrara and Henzel, 1989; Senger et al., 1983; Unemori et al., 1992).

Vascular remodeling in brain metastases

Since the original observation of Weidner (1998), many investigators suggest that the mean vessel density (MVD) within or at the periphery of neoplasms correlates with the aggressiveness of the disease. This generalization, however, does not extend to brain metastases. Circulating tumor cells that reach the brain vasculature are initially aligned along existing blood vessels. Enlargement of the tumor lesions is associated with dilation (ectasia) of blood vessels. Murine melanoma fibrosarcoma, human colon carcinoma, and human lung adenocarcinoma cells produced well-demarcated lesions in the brain parenchyma of nude mice (Fidler et al., 2002). These metastases contained large blood vessels with dilated lumens. Moreover, the lumen of blood vessels on the periphery of these experimental brain metastases was also dilated. The MVD within these lesions was 15-20 times lower than the MVD in the surrounding uninvolved brain parenchyma or brain parenchyma of normal uninjected nude mice. The experimental brain metastases produced by the colon or lung cancer cells contained blood vessels with dilated lumens, and large metastases contained large blood vessels with transverse bridges and multilumen structures that were lined with $CD31^+$ endothelial cells (Fidler et al., 2002). The formation of multilumen vessels, originally described as vascular hyperplasia (Feigin et al., 1958), is thought to be a form of vascular remodeling from a vessel with large lumen to smaller size vessels (Holash et al., 1999; Nagy et al., 1995; Patan et al., 1996) by a process called nonsprouting angiogenesis, *i.e.*, new blood vessels are formed by the "splitting" of preexisting dilated blood vessels (Patan, 1998).

The dilation of blood vessel lumen in both the experimental brain metastases and surgical specimens of human lung cancer brain metastases was associated with the division of endothelial cells. We base this conclusion on the data showing that $BrdU^+$, $CD31^+$ cells were located within the walls of the vessel among nondividing endothelial cells. The observed vessel dilation, *i.e.,* angioectasia, therefore, did not occur merely by stretching of the blood vessel wall but rather as a consequence of endothelial cell division within the wall of the blood vessel (Fidler et al., 2002).

The progressive growth of experimental brain tumors and experimental brain metastases (Brown and Giaccia, 1998) is dependent on expression of VEGF/VPF. We base this conclusion on the data from experiments where cancer cell lines were injected into the carotid artery of nude mice (Yano et al., 2000). Although this injection bypassed the initial steps of metastasis (separation from the primary neoplasm, invasion, and release into blood vessels or lymphatics), to produce brain metastases, all subsequent steps in the process (arrest in brain capillary bed, extravasation, growth, and angiogenesis) had to occur. The differences in growth pattern of experimental brain metastasis did not correlate with the initial arrest of tumor cells (measured by radiolabeling of tumor cells with ^{125}I-IdUrd) or collagenase activity (measured by gelatin zymography) but, rather, due to unique pattern of vascularization (Brown and Giaccia, 1998).

Human colon carcinoma (KM12SM) and lung adenocarcinoma (PC14PE6, PC14Br) cells produced large, fast-growing parenchymal brain metastases in nude mice (Yano et al., 2000), whereas lung squamous cell carcinoma (H226) and renal cell carcinoma (SN12PM6) cells produced only a few slow-growing brain metastases. Rapidly progressing brain metastases contained many enlarged blood vessels with transluminal bridges of endothelial cell processes (the hallmark of nonsprouting angiogenesis). The expression of VEGF mRNA and protein by the tumor cells directly correlated with nonsprouting angiogenesis and growth of brain metastasis. Causal evidence for

the essential role of VEGF in these processes was provided by transfecting PC14PE6 and KM12SM cells with antisense-VEGF165 gene, which significantly decreased the incidence of brain metastasis and enlarged blood vessels. In contrast, transfection of H226 human lung squamous carcinoma cells with sense-VEGF121 or sense-VEGF165 neither enhanced nor inhibited formation of brain metastases (Yano et al., 2000). Collectively, the results indicate that VEGF expression is necessary but not sufficient for the production of brain metastasis and nonsprouting angiogenesis and that the inhibition of VEGF represents an important therapeutic target (Stewart et al., 1987; Yano et al., 2000).

Location of dividing and apoptotic tumor cells in relation to the vasculature

The diffusion coefficient of oxygen within tissues is on the order of 150-200 μm (Jain, 2001; Russ, 1989; Tannock, 1968). Since cell viability is dependent on oxygen, we determined whether the location of dividing or apoptotic tumor cells within brain metastases correlates with their distance from the nearest blood vessel. Because the growth of discrete-focal experimental brain metastases was associated with fewer but larger blood vessels per unit area, we determined whether the proximity of tumor cells to blood vessels correlated with DNA synthesis by double-labeling tissue sections for BrdU (cell division) and CD31 reactivity (endothelium). The spatial distribution of $BrdU^+$ nuclei of $CD31^-$ cells relative to the nearest blood vessel was determined using the Euclidean distance map (EDM) (Chalkley, 1943; Gavrieli et al., 1992). Since actively synthesizing endothelial cells stained for both CD31 and BrdU, they did not affect the analysis. In autochthonous human lung cancer, brain metastasis dividing cells were located mostly within 75 μm of the nearest vessel (Fidler et al., 2002).

The distance of apoptotic cells from the nearest blood vessel was determined by an end-labeling assay (Tdt-mediated dUTP-biotin nick-end labeling, or TUNEL) (Russ, 1995). Apoptotic cells ($TUNEL^+$) in autochthonous human lung cancer brain metastases were mostly located 160-170 μm from the nearest blood vessel (Fidler et al., 2002). Collectively, the data demonstrate that the location of both dividing and apoptotic tumor cells within clinical specimen of brain metastases correlates with the diffusion coefficient of oxygen within tissues (Jain, 2001; Russ, 1989; Tannock, 1968).

The blood-brain barrier in brain metastasis

The microvasculature of the brain parenchyma is lined by a continuous, nonfenestrated endothelium with tight junctions and little pinocytic vesicle activity (Felgenhauer, 1986; Gregoire, 1989; Johansson, 1990). This structure, designated the blood-brain barrier (BBB), limits the entrance of circulating macromolecules into the brain parenchyma. The BBB and the lack of a lymphatic system are responsible for maintaining the brain as an immunologically privileged site (Shapiro and Shapiro, 1986) and protecting the brain against the entry of most drugs and invasion by microorganisms (Stewart et al., 1987). The BBB does not prevent the entry of circulating metastatic cells into the brain parenchyma. In fact, some but not all neoplastic cells can affect the integrity of this structure (Schlingemann et al., 1990; Zagzag et al., 1989; Zuelch, 1986).

In general, primary brain neoplasms and brain metastases are resistant to treatment by most chemotherapeutic drugs (Greig et al., 1990; Stewart et al., 1987), and this resistance has been attributed to the inability of drugs to cross the BBB (Zhang et al., 1992). We investigated the functional viability of the BBB in an experimental model of brain metastases (Holash et al., 1999; Schackert and Fidler, 1988b). Different human tumor cell lines were injected into the internal carotid artery of nude mice (Tomlinson, 1987). To study the permeability of the BBB, we chose sodium fluorescein (Felgenhauer, 1986).

Astrocytes protect tumor cells from chemotherapy

Astrocytes maintain homeostasis of the brain microenvironment (Abbott et al., 2006; Sofroneiw, 2005) by transporting various nutrients from the circulation to the neurons, participating in neural signal transduction, and buffering the ionic balance of the extracellular matrix (Allen and Barres, 2009; Bullock et al., 2005; Fields and Stevens-Graham, 2002; Miller, 2005). Under pathological conditions, astrocytes became activated, as indicated by their upregulation of glial fibrillary acidic protein (GFAP) (Crooks et al., 1991). Since these reactive astrocytes have been shown to protect neurons from injury induced apoptosis (Chen et al., 2005; Mahesh et al., 2006; Siofroniew, 2005), we determined whether reactive astrocytes can also protect tumor cells in brain metastases from cytotoxicity induced by chemotherapeutic drugs. To test this hypothesis, we studied the sensitivity of different tumor cells to chemotherapeutic agents when co-cultured with mouse astrocytes or fibroblasts. We utilized an immortalized astrocyte cell line derived from the brain of *H-2K^b-ts*A58 mice (Langley et al., 2009) and co-cultured these astrocytes with tumor cells at a ratio of 1:1. A scanning electron microscopic examination revealed that the astrocytes formed direct contacts with tumor cells through multiple podia.

Next, we evaluated chemotherapy-induced apoptosis in tumor cells in the absence and presence of astrocytes. Co-culture with astrocytes dramatically reduced 5-fluorouracil (5-FU)-induced apoptosis in the human tumor cells. This protection was not specific to the chemotherapeutic agent because astrocytes demonstrated similar reductions in cytotoxicity in tumor cells treated with Cisplatin. Astrocytes cultured alone or co-cultured with tumor cells did not undergo apoptosis when incubated with the chemotherapeutic agents under similar conditions. To determine whether protection by astrocytes required secreted factors or direct physical contact, we repeated the experiments but separated the astrocytes from tumor cells by a trans-well membrane (0.4 μm pore size). Under these conditions, the astrocytes failed to protect tumor cells from chemotherapy-induced apoptosis. Substituting murine NIH3T3 fibroblasts for astrocytes in the co-culture experiments failed to provide protection. This chemo-protective nature of astrocytes was also demonstrated in various human melanoma cells (Lin et al., 2010), human breast cancer cells, and human lung cancer cells (Kim et al., 2010).

Resistance to chemotherapy is a major cause of death in patients with brain metastasis. This resistance has been attributed to the impermeable nature of the BBB and the expression of P-glycoprotein by metastatic cells. Our data provide a novel alternate mechanism that requires direct contact between activated astrocytes and tumor cells in the brain tumor microenvironment. These data underscore the ability of metastatic brain tumors to exploit the neuroprotective properties of resident astrocytes for their own survival.

Conclusion and perspective

More than 40% of patients with lung cancer and breast cancer develop brain metastasis. With improved local control and therapy of metastasis to visceral organs, the morbidity and mortality due to late diagnosed brain metastasis are projected to rise. The median survival for untreated patients is 1-2 months, which may be extended to 6 months with radiotherapy and chemo-

therapy.

The development of a relevant mouse model for the establishment and growth of brain metastasis has been instrumental for studies of the biology and therapy of this most feared consequence of cancer. Injection of human tumor cells into the internal carotid artery of mice produces experimental metastases in specific regions of the brain that are not due to patterns of initial cell arrest, motility, or invasiveness, but rather to the ability of metastatic tumor cells to grow. Immunohistochemical and morphometric analyses demonstrate that the density of blood vessels within experimental metastases in brains of nude mice or clinical specimen of human lung cancer brain metastases is lower than that in the adjacent tumor-free brain parenchyma. However, brain metastasis-associated blood vessels are dilated and contain numerous dividing endothelial cells. Immunohistochemical analysis also reveals that tumor cells located less than 100 μm from a blood vessel are viable, whereas more distant tumor cells undergo apoptosis. Tumor cells within brain metastasis produce VEGF which induces permeability in adjacent vessels. The BBB in metastases that are larger than 0.25 mm in diameter is leaky. The lesions, nevertheless, are resistant to chemotherapeutic drugs.

The respected "seed and soil" hypothesis suggests that the outcome of metastasis depends on the interaction between unique tumor cells and the specific organ microenvironment. The demonstration that activated astrocytes whose physiological role is to protect neurons from toxic substances can be exploited by tumor cells for protection from chemotherapeutic drugs suggests new approaches to the treatment of this fatal disease.

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