Review

The role of the organ microenvironment in brain metastasis

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Abstract

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 surgery, radiotherapy, and chemotherapy. The development of a relevant mouse model for the establishment and growth of brain metastasis has advanced our understanding of the biology and therapy of this most feared consequence of cancer. Injection of murine or 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 exploit homeostatic mechanisms and proliferate. Immunohistochemical and morphometric analyses demonstrate that the density of blood vessels within experimental metastases in brains of mice or in 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. Metastases in the brain are resistant to chemotherapeutic drugs.

The venerable “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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1. Introduction

In the United States, more than 40% of cancer patients develop metastasis to the brain [1]. The most frequent metastasis to the brain occurs in patients with lung, breast, melanoma, renal, and colorectal tumors [2]. Once metastasis to the brain is diagnosed, the median survival of untreated patients is 1–2 months. In patients treated with surgery, chemotherapy, and radiation therapy, the median survival can be extended to only 4–6 months. The poor prognosis in these patients is due primarily to resistance to chemotherapy and the recurrent growth of tumors at the site of resected lesions, as well as the development of metastases in other areas of the brain [3–5]. Improvements in treatment of brain metastasis can only come from a better understanding of the biology of these lesions.

In 1889, Stephen Paget questioned whether the organ distribution of metastases produced by different human neoplasms was due to chance and analyzed 735 autopsy records of women with breast cancer. He 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 [6]. In 1928, James 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 [7]. 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 into muscles, but not in renal tissue implanted as a control or at the

Abbreviations: BBB, blood–brain barrier; MVD, mean vessel density; VEGF, vascular endothelial growth factor.

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site of surgical trauma. Injection of radioactive-labeled tumor cells revealed that just as many tumor cells reached the lung and kidney implants, but since metastasis developed only in the natural lung and implanted lung fragment, the results confirmed that sites of metastasis are determined by the characteristics of the neoplastic cells and the microenvironment of the host tissue [8].

The introduction of peritoneovenous shunts for the palliation of ascites in women with progressive ovarian cancer provided the opportunity to study some of the factors that affect metastatic spread in humans. Good palliation with minimal complications was reported for many patients. The autopsy findings in 15 patients substantiated the clinical observations that the shunts did not increase the risk of metastasis. Actually, despite continuous entry of millions of viable tumor cells into the circulation, metastases in the lung (the first capillary bed encountered) were rare [9].

A current definition of the “seed and soil” hypothesis consists of three principles. Primary tumors in general and metastatic lesions in particular are biologically heterogeneous and contain multiple cell populations with diverse characteristics of karyotype, growth rate, cell surface receptors, antigenicity, immunogenenicity, hormone receptors, sensitivity to cytotoxic drugs, production of extracellular matrix proteins, adhesion molecules, angiogenic potential, invasiveness, and metastatic potential [10–13]. Second, the process of metastasis is highly selective for cells that can complete all of the steps in the process [14,15]. Metastases can have a clonal origin, and different metastases can originate from the proliferation of different single cells [16,17]. Third, the outcome of metastasis depends on multiple interactions of metastatic cells with host homeostatic mechanisms that include the organ microenvironment which tumor cells exploit for their own gain [14,18]. Metastasis to the brain is a prime example.

2. In vivo models to study the biology of brain metastasis

To study 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 tumors in the brain and especially brain metastases [19–21]. However, the injection of cells into the carotid artery of nude mice simulates the hematogenous spread of tumor emboli to the brain, i.e., the release of tumor cells into the circulation, arrest in capillaries, penetration and extravasation into the brain parenchyma and growth of tumor cells in the brain tissue.

3. The origin of brain metastases

Early clinical observations have suggested that brain metastases produced by many solid tumors occur late in the disease [22]. These findings questioned whether brain metastases are produced by cells originating from the primary neoplasm or from 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” [23]. For the pathogenesis of brain metastasis, this is not an academic issue. If metastasis to the brain occurs by “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 specific metastatic clones from the primary lesion [24], then prophylactic dissection of extracranial metastases may not prevent the formation of brain metastasis.

We examined 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 of patients as well as the metastatic potential of three variants of the mouse K-1735 melanoma [25]. Tumorigenicity and metastatic potential were determined in nude mice (for the human melanoma cell lines) or in syngeneic mice (for the K-1735 cell variants). The ability to form lesions in the brain was tested by direct injection of tumor cells into the internal carotid artery. For the human melanomas, cells derived from patients’ lymph node metastasis were more tumorigenic (s.c. or lung) and metastatic in nude mice than melanoma cells isolated from clinical brain metastases. In the K-1735 mouse melanoma system, the different melanoma cell lines showed similar frequencies of tumor take in the brain, regardless of tumor growth in other organs. These results suggested that since cell lines derived from metastases in the brain were not more malignant than cells isolated from the lymph node or lung metastases, the data implied that the colonization of brain tissue is not necessarily the final stage of a metastatic cascade [25].

4. Unique patterns of melanoma metastasis to the brain

Malignant melanoma will produce metastases in the brain of most patients [1,2]. Of these brain metastases, 49% are intra-parenchymal, 22% are leptomeningeal, and 32% are dural [26,27]. To determine whether mouse melanoma cell lines also produce site-specific brain metastasis, we used cells from the K-1735 melanoma syngeneic to C3H/HeN mice [19] and the B16 melanoma syngeneic to C57BL/6 [28,29]. Regardless of whether we injected the melanoma cells into the external or internal carotid arteries, the K-1735 cells produced melanotic lesions only in the brain parenchyma of all injected mice, and the B16 cells produced lesions only in the meninges and ventricles in the brains of all injected mice [20].

Studies on the distribution and fate of hematogenously disseminated radiolabeled tumor cells have concluded that tumor cells can reach the microvasculature of many organs, but the growth of the arrested cells into clinically relevant metastases occurs in only some [30]. Subsequent to the injection of radiolabeled murine melanoma cells into the internal carotid artery, we found that most cells were trapped in the vasculature of the brain. For both the murine melanomas, only a few cells reached the meninges. The K-1735 cells failed to proliferate at this site, whereas the B16 cells grew there rapidly [20]. These data confirmed that initial tumor cell arrest in the microvasculature did not correlate with the development of progressively growing lesions [30,8].

We have also analyzed the ability of human melanoma cells from 8 different lines established from subcutaneous, lymph node, or brain metastases of different patients to cross the blood–brain barrier and proliferate in the brain of nude mice [31,32]. Cells from all but one human melanoma line produced experimental brain metastases following injection into the carotid artery. While tumor lesions were found in the meninges, ventricle, and parenchyma, each melanoma showed a slightly different pattern of growth. Of interest were the findings that two cell lines derived from two different brain parenchyma metastases from two different patients showed a preference for growth in the brain parenchyma of nude mice. The cell lines derived from lymph node or subcutaneous metastases of patients grew more frequently in the meninges or ventricles than in the brain parenchyma of the nude mouse.

The biological behavior of different human melanoma cell lines and cells isolated from fresh surgical specimens of cutaneous melanoma, lymph node metastases, and brain metastases was determined subsequent to direct intracerebral implantation in
nude mice [32]. Melanoma cells isolated from cutaneous lesions or lymph node metastases produced leptomeningeal disease but did not invade the brain parenchyma, whereas cells isolated from brain metastases produced leptomeningeal disease and infiltrative intraparenchymal lesions. The ability of human melanoma cells to grow in the brain parenchyma was inversely correlated with their sensitivity to the antiproliferative effects of TGF-β2 [22].

5. 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 intracarotid injection, human carcinoma cells from all primary neoplasms grew in the brain of nude mice, thus demonstrating that if carcinoma cells can reach the brain parenchyma, they can proliferate in the brain. Tumor growth occurred more frequently in the parenchyma than in other regions of the brain. Moreover, the growth of tumor cells in the brain parenchyma revealed that some cells from all of the carcinoma cell lines examined were able to cross the blood–brain barrier [33,34].

6. Vascular remodeling in brain metastases

Since the original observation of Weidner [35], many investigators have suggested 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 [15]. These metastases contain few but large blood vessels with dilated lumens. The lumens of blood vessels on the periphery of these experimental brain metastases was also diluted. The MVD within these lesions was 15–20 times lower than the MVD in the surrounding uninvoluted brain parenchyma or brain parenchyma of normal un.injected nude mice. The experimental brain metastases produced by colon carcinoma or lung carcinoma 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 [15]. The formation of multilumen vessels is thought to be a form of vascular remodeling from a vessel with large lumen to smaller size vessels by a process called nonsprouting angiogenesis, i.e., new blood vessels are formed by the “splitting” of preexisting dilated blood vessels [36].

We observed dilation of blood vessel lumen in both experimental brain metastases and surgical specimens of human lung cancer brain metastases. This dilation 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 [15].

The progressive growth of experimental brain tumors and experimental brain metastases [37] is dependent on expression of vascular endothelial growth factor/vascular permeability factor (VEGF/VPF). We base this conclusion on the data from experiments where different cancer cell lines were injected into the carotid artery of nude mice [38]. Once again, 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 125I-labeled Urd) or collagenase activity (measured by gelatin zymography) but, rather, due to unique pattern of vascularization [37].

The intracarotid injection of human colon carcinoma and lung adenocarcinoma cells produced large, fast-growing parenchymal brain metastases in nude mice [38], whereas the intracarotid injection of human lung squamous cell carcinoma and human renal cell carcinoma cells produced only a few slow-growing lesions in the brain of nude mice. Rapidly progressing brain metastases contained many enlarged blood vessels with transmural 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 human lung and human colon carcinoma cells with antisense-VEGF165 gene, which significantly decreased the incidence of brain metastasis and enlarged blood vessels. In contrast, transfection of human lung squamous carcinoma cells with sense-VEGF121 or sense-VEGF165 neither enhanced nor inhibited formation of brain metastases [38]. Collectively, the results indicate that VEGF expression is necessary but not sufficient for the production of brain metastasis and non-sprouting angiogenesis and that the inhibition of VEGF represents an important therapeutic target [38–41].

7. 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 [42–44]. 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) [45]. 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 [15].

The distance of apoptotic cells from the nearest blood vessel was determined by an end-labeling assay [46]. Apoptotic cells (TUNEL+) in autochthonous human lung cancer brain metastases were mostly located 160–170 μm from the nearest blood vessel [15]. 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 [42–44].

8. Brain metastasis and the blood–brain barrier

The microvasculature of the brain parenchyma is lined by a continuous, nonfenestrated endothelium with tight junctions and little pinocytic vesicle activity [47–50]. This structure, designated as the blood–brain barrier (BBB), limits the entrance of circulating macromolecules into the brain parenchyma. The blood–brain barrier and the lack of a lymphatic system are responsible for maintaining the
endothelial pinocytosis, opening of the interendothelial tight junctions, and the integrity of the BBB is altered in primary brain tumors and in metastases [53–60].

Primary brain tumors and brain metastases are resistant to treatment by most chemotherapeutic drugs [51,52], and this resistance has been attributed to the inability of drugs to cross the BBB [4,5,61,62]. However, since this structure is morphologically, biochemically, and functionally heterogeneous in different regions of the brain [63–65], its relationship to the failure to treat brain metastases with chemotherapeutic drugs is questionable. We investigated the functional viability of the blood–brain barrier in experimental system described above to study the establishment, progression, and therapy of brain metastases [19–21,66]. Eight different human tumor cell lines were inoculated into the internal carotid artery of nude mice. The tumors produced lesions in different regions of the brain and the pattern of the lesions varied from diffuse growths to solitary lesions with well-defined margins. Of several molecular tracers used to study the permeability of the BBB, we chose sodium fluorescein. Despite its low molecular weight (MW 376), this hydrophilic molecule is excluded from the brain by an intact blood–brain barrier [67,68]. Sodium fluorescein is not sensitive to minor or transient changes in blood–brain barrier permeability, and unlike horseradish peroxidase, it is not transported into brain tissue by nonspecific endocytosis [69]. This molecule is therefore most suitable for studies of blood–brain barrier functions in brain metastases.

Before studying the function of the blood–brain barrier in such brain lesions, we ruled out that the procedure of intracarotid injection, which is followed by ligation of the artery, or the entry of a bolus of tumor cells into the brain damages the endothelial cells of the cerebrovascular system and, thus, change the permeability of the blood–brain barrier [15,70]. Leakage through the BBB may be due to endothelial alterations brought about by tumor cells in the perivascular space [71]. Several ultrastructural studies concluded that brain tumors disrupt adjacent endothelium [50,72]. In our study with eight different human tumor cell lines, the lesions in the brain parenchyma were either well demarcated with well-defined margins or diffuse lesions throughout a region of the parenchyma. We found that the solitary well-defined lesions had a lower density of blood vessels than normal brain tissue. The blood–brain barrier is known to be permeable in ischemic regions of the brain where increased cytoplasmic processes of oligodendrocytes are likely to be important in maintaining a functional BBB. A growing tumor mass may disturb this interaction especially if it depends on contact between astrocytes and endothelial cells. In any event, the normal brain tissue interspersed among the small tumor clusters or surrounding small tumor lesions might be responsible for the normal function of the BBB.

Our results indicate that the permeability of the BBB in experimental brain metastases produced by different human tumor cells after injection into the carotid artery of nude mice was determined by the location of tumor lesions in the brain and by the size and pattern of growth of the lesions. Since the BBB is not intact in experimental brain metastases that exceed 0.2 mm², the resistance to chemotherapy must be due to other mechanisms.

9. Astrocytes protect tumor cells from chemotherapy

Astrocytes maintain homeostasis of the brain microenvironment [75,76] by participating in neural signal transduction, transporting various nutrients from the circulation to the neurons, and buffering the ionic balance of the extracellular matrix [77–80]. Under pathological conditions, astrocytes become activated, and the cells upregulate expression of glial fibrillary acidic protein (GFAP) [81]. Since these reactive astrocytes have been shown to protect neurons from injury induced apoptosis [75,82,83], we determined whether reactive astrocytes (GFAP⁺) 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 cultured alone or co-cultured with mouse astrocytes or fibroblasts. We utilized an immortalized astrocyte cell line derived from the brain of H-2Kb-tsA58 mice [84] 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.

Chemotherapy-induced apoptosis in tumor cells was evaluated in the absence and presence of astrocytes or fibroblasts. Co-culture with astrocytes significantly reduced 5-fluorouracil (5-FU) or cisplatinum-induced apoptosis in the human or murine tumor cells. 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 using astrocytes separated 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 did not provide protection from chemotherapy. This chemoprotective nature of astrocytes was also demonstrated in various human melanoma cells [85], human breast cancer cells, and human lung cancer cells [86].

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 clearly demonstrate that metastatic cells exploit homeostatic mechanisms to their own advantage and that therapy of brain metastases must be directed against both the tumor cells (“seed”) and the organ microenvironment (“soil”) [6,14].


