Chapter 7

Role of Brain Microenvironment in Brain Metastases

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Abstract: The development of brain metastasis portends a grave prognosis for patients with systemic cancer. Efforts to alter the course of this disease have been hampered by a poor understanding of the biology of the metastatic process. Recent insights into the biologic determinants of this process aided by advances in molecular biology and biotechnology have altered the basic concepts of our understanding of how cancer cells metastasize to distant organs. These findings have validated and extended the "seed and soil" hypothesis emphasizing a critical role for the microenvironment of the target organ in the development of metastatic lesions. The brain microenvironment has unique characteristics that distinguish it from other organs of the body. Hence, therapeutic strategies to target the interaction between the metastatic tumor cell and the brain require a clear understanding of the molecular and anatomic features that influence this process. Recent studies have revealed an intricate and often facilitatory interaction between these elements of the brain metastatic process. These findings may allow the development of targeted therapies that in combination with therapeutic strategies against systemic malignancies hold promise to improve the prognosis of patients with brain metastases.

Key words: Brain, metastasis, microenvironment, molecular mediators

1. INTRODUCTION

Brain metastases are the most common malignancies affecting the nervous system, and their incidence far outnumbers the incidence of primary brain tumors (1). In autopsy series, intracranial metastases (symptomatic or undetected) have been demonstrated in 24% of all cancer patients examined (2). The disease confers significant mortality and morbidity. Median survival from the time of detection is 4 weeks in the absence of therapeutic intervention(s), with death resulting from intracranial disease progression. (3, 4) Even with advances in current treatments, the overall median survival remains in the range of 3-6 months. (5-7) The morbidity associated with brain metastases results from the progressive development of neurologic and systemic symptoms (8). Many therapeutic approaches have attempted to alter the course of the disease, but they have only minimally affected the overall course of the malignancy and the prognosis of the patient. The privileged status of the brain created by the blood–brain barrier (BBB), the co-existence of progressive systemic and intracranial disease that can obscure morbidity due to brain disease, and the limited understanding of the biology of metastatic disease processes have hindered the development of meaningful therapeutic advances. Understanding the biology of the metastatic process has in part been limited by difficulties in obtaining brain metastatic tumor tissue, which would enable researchers to study the determinants underlying the biologic behavior of brain metastases. There is also a paucity of investigators whose preclinical and translational studies are predominantly focused on understanding the brain metastatic process. In

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addition, most clinical trials of new anticancer agents in humans exclude from enrollment patients with brain metastases, which precludes appreciation of the effects of the agents being assessed against this disease process. Although in some malignancies the occurrence of brain metastasis is an early event, possibly due to intrinsic biologic characteristics of the primary tumor, in most cases, the appearance of metastatic lesions in the brain occurs only in the late stages of disease. The progressive increase in overall tumor burden overwhelms natural biologic boundaries that normally insulate the brain from such events. Because of the overlapping effects of systemic and intracranial disease, clinical trial designs are required to be increasingly complex and the outcomes are difficult to measure. Furthermore, recent advances in molecular pharmacotherapeutics and biotechnology have been translated into improved control of the underlying systemic disease, making it increasingly likely that disease sheltered in protected sites such as the brain could become a more relevant determinant of patient prognosis than the primary disease. It is thus imperative to gain insights into the biology of brain metastasis so that new and rational therapeutic approaches can be developed for controlling this disease.

2. EVOLUTION IN THE CONCEPTS OF THE METASTATIC PROCESS

The initial concept was that metastasis develops from tumor cells that are shed from a primary lesion into the circulation, followed by passive transfer of the cells until they are arrested in the capillaries of target organs where they establish new disease foci (9). It is possible that in the past the lack of effective treatments resulted in uncontrolled disease progression, which rapidly increased the overall disease burden. In such a setting, widespread metastases are common and organ selectivity may be less apparent. Generally, these lesions localize to the gray-white matter junction, most frequently in watershed regions of the brain's blood supply (10, 11). This pathologic pattern of distribution is invoked to support the common notion that the spread of brain metastases is primarily hematogenic. However, more than a century ago, it was observed that the occurrence of metastases did not follow simple rules based on anatomy or blood supply. The inference was that factors critical to the development of metastases were related to the tissue of origin as well as to the target tissue (12). For brain metastases, this idea evolved into the intriguing theory that not only are specific cells in the primary tumor primed to metastasize to the brain but that there may also be cooperation between metastatic tumor cells and the brain microenvironment that helps to establish metastatic tumor foci in the brain. This concept has been strengthened by the observation that some malignancies have a higher predilection than others to metastasize to the brain. It is now well accepted that brain metastatic disease is the result of several combined factors, including the tissue of origin of the primary tumor, biologic factors related to the phenotype of the involved tumor cells, and the brain microenvironment. Together, these factors strongly influence host tissue-tumor cell interactions, and anatomic and physiologic mediators that regulate the transport and physical arrest of metastatic cells. A better understanding of the biology of this process has opened the door for developing targeted therapeutic interventions, an area of interest that has been intensively investigated in recent years. This chapter is an overview of some of the recent advances in the field of brain tumor metastasis, with reference to the various molecular factors relevant to this process, and it also examines how a better understanding of these factors is helping in the effort to conceptualize and develop novel therapeutic approaches for more effectively managing brain metastases.

3. THE BRAIN MICROENVIRONMENT – RELEVANCE TO METASTASIS

Based on the concept that "the distribution of the secondary growths is not a matter of chance", Stephen Paget proposed a "seed and soil" hypothesis, which suggested that intrinsic characteristics of both the metastasizing cells and the host tissue were critical to the establishment and advancement of metastatic disease (12). Clinical

observation supports the concept that malignancies have a predilection to metastasize to specific target organs and that the number and frequency of occurrence of such lesions vary widely among individuals. Importantly, the distribution of metastatic lesions in various organs in the body is not proportional either to their total vasculature or total endothelial surface area. These observations suggest the existence of specific intercellular interactions due to the biology of the involved tumor cells as well as from the "readiness" of the microenvironment in the target organ to "receive" these cells. Preclinical studies of the interactions between tumor cells and their microenvironment support this mutual dependence between individual cells and individual physiologic environments. Such early concepts evolved into a more comprehensive view of the metastatic process, which now incorporates *mechanical factors* such as blood flow sludging, platelet-related interactions, *physiologic factors* such as hemodynamic changes, pH regulation, oxygen concentration, and metabolic demand, *biologic factors* such as expression of adhesion molecules and receptors in the target organ, and *molecular characteristics* of metastatic tumor cells related to their intrinsic biology and their tissue of origin. Despite the apparently insurmountable nature of these complex interactions for developing therapeutic approaches, insights into the biology of the metastatic process are facilitating the development of targeted approaches to treatment.

4. THE BRAIN MICROENVIRONMENT-STRUCTURAL AND FUNCTIONAL CONSIDERATIONS

Overview. As is true for metastatic disease in general, metastases to the brain are influenced by the anatomy and physiology of the target organ. The lymphatic system that places a significant role in metastasis in other organs is absent in the brain. Direct extension of tumor into the brain from adjacent structures is an unusual occurrence. When it occurs, it often results in compression and displacement of the brain rather than infiltration. Thus, most metastatic cells reach the brain through its rich blood supply via extensive capillary beds that serve areas of high metabolic activity. The subset of these cells that form tumor foci in the brain traverse the microvasculature and eventually arrest in the terminal branches of the small capillaries supplying the brain by various physiologic and molecular mechanisms discussed in detail later in this section. The cells traverse the vascular endothelium, cross the blood–brain barrier, and migrate by following specific microenvironmental cues that determine the final site of tumor growth. In brain, tumor cells proliferate to form a nidus that continuously interacts with the brain microenvironment. The small metastatic mass continues to proliferate until it reaches a critical mass beyond which its oxygen requirement cannot be sustained by diffusion and leads to progressive hypoxia. Cells susceptible to these insults may perish whereas others resort to anaerobic metabolism, resulting in the generation of acidosis and, subsequently, necrosis. These events generate angiogenic signals that promote the growth and establishment of a fresh network of collateral blood vessels, which then supply the mass. This event triggers renewed proliferation in the tumor and changes in vascular permeability. The resultant extensive edema eventually causes displacement, infiltration and local destruction of the brain tissue. Clinical symptoms ensue because of these local effects, declaring the presence of the metastatic lesion.

Vasculature of the Brain. The blood supply to the brain is derived from the "anterior'" circulation comprised of the two internal carotid arteries, and the "posterior" circulation formed by the two vertebral arteries that communicate at the base of the brain via the circle of Willis and divide into numerous branches within the brain. The middle cerebral arteries supplies the frontoparietotemporal regions and the anterior cerebral arteries supply the medial frontoparietal of the brain above the tentorium; the vertebral arteries, on the other hand, enter the posterior fossa, supplying the brainstem and cerebellum as well as parts of the parieto-occipital and medial temporal regions of the brain. The main arteries enter the subarachnoid space and

branch extensively, forming rich anastamoses at the pial surface before forming pial vessels and arterioles that penetrate the brain substance. These long and medullary arteries traverse through the cortex and penetrate the subjacent white matter without inter-communication, thus forming small independent vascular systems within the cortex. The terminal branches of these blood vessels are the capillaries (< 10μm in diameter) that form a rich network of anastamoses in the white matter (13). It has been estimated that the surface area of the brain microvasculature is approximately 100 cm^2 per gram of tissue (14). Subcortical arteries that enter the white matter coil, loop and spiral in large adventitious spaces, giving off few neocortical branches, and dispersing within the white matter. These loops may have areas of turbid flow and potentially function as mechanical traps for circulating tumor cells (15). After numerous anastamoses become established among capillaries within the substance of the brain, draining venules and veins form. These subsequently converge into cerebral venous sinuses that exit the brain via the jugular veins. In addition to the anatomic features described above, the brain microvasculature is subject to elaborate physiologic controls based on local metabolic demands and is capable of modulating flow in response to such stimuli.

The Blood–Brain Barrier. The endothelium lining the blood vessels forms the first, and possibly the most significant, barrier that a metastatic cell encounters upon entering the nervous system. The BBB refers to the highly specialized boundary between blood and the brain substance and is composed primarily of nonleaky-type tight junctions between capillary endothelial cells. These junctions are reinforced by pericytes, astrocytic foot processes, and joint basal laminae. These components function together as a complex filtering mechanism that mechanically restricts large molecules, infectious agents, and cells from infiltrating the substance of the brain. In addition, this system dynamically controls the entry of diverse molecules, drugs or toxins through receptor systems, specialized channels, and via other poorly understood active filtering processes.

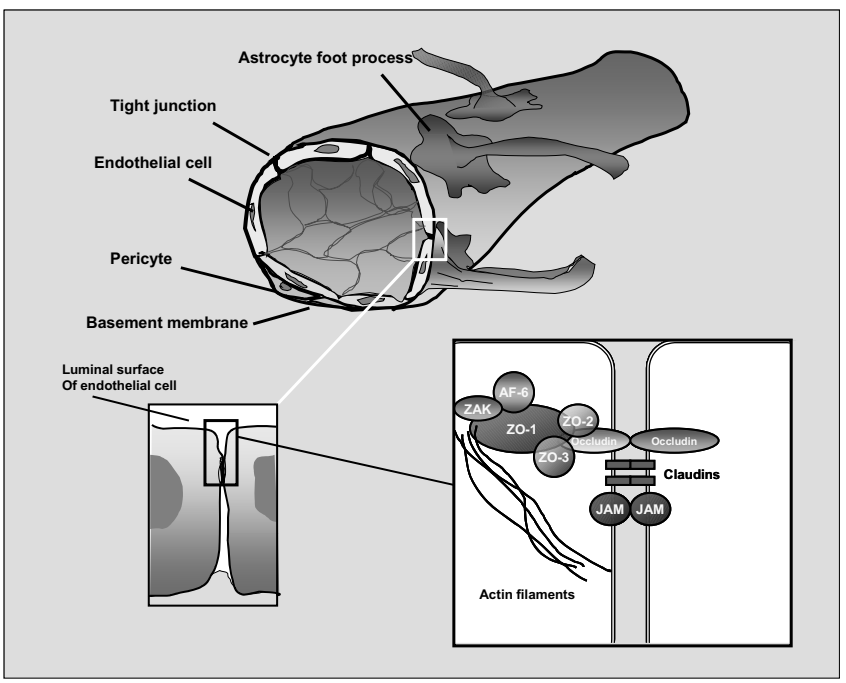

Figure 1. Ultrastructure of the Blood-Brain Barrier.

The molecular composition of the BBB is not fully understood but recent studies have provided insights into the specialized nature of the molecular architecture and dynamics of this unique barrier (Figure 1). The endothelial cells, which are the main cellular component of the BBB, have characteristic intercellular regions of apposing contact called tight junctions (zona occludens) that are relatively specific to the BBB and provide regions of high electric resistance that result in low permeability (16). By their presence in the apical (luminal) regions of the endothelial cell-cell contact zone, they form a continuous paracellular barrier, sealing the endothelial surface and forming the most restrictive element of the BBB. They are composed of a complex combination of proteins, including several transmembrane proteins such as claudin, occludin, and junctional adhesion molecules (JAM) that are organized around an actin cytoskeletal matrix (17, 18). Being located in the apical regions of the intercellular clefts towards the luminal surface of the endothelial cell, these proteins can interact with various adhesion molecules present in circulating cells, and regulate adhesion and migration of leukocytes, platelets and possibly tumor cells. On the intracellular side, the transmembrane proteins in turn intimately interact with the cytoskeletal proteins, including actin filaments and several accessory cytoplasmic proteins such as zona occludens-1and 2 (ZO-1 and ZO-2) proteins, cingulin, 7H6, Rap and AF-6 proteins, which are organized around scaffolding proteins (19). In addition to these structural considerations, it is known that phosphorylation of the transmembrane and accessory proteins can rapidly regulate tight junction function and hence affect permeability across the BBB. For example, phosphorylation and de-phosphorylation of claudin changes the structural integrity of the tight junction, usually improving the assembly of the junction. It has also been noted that tyrosine phosphorylation of existing tight junction proteins can decrease occludin expression, leading to increased permeability (20). Conversely, in mature junctions and well-formed cell-cell contacts, there is reduced tyrosine phosphorylation of proteins involved in cell-to-cell contact (21). These regulatory processes are important in modifying the integrity of the BBB and putatively influence its interaction with components in the blood such as metastatic tumor cells.

Under physiologic conditions, cellular components of the blood cannot traverse the brain microvascular endothelium. However, pathologic alterations of the BBB can break down its barrier functions, allowing proinflammatory mediators, such as reactive oxygen species and cytokines, to induce upregulation of surface adhesion molecules such as PECAM-1, E-selectin, and ICAM-1. More adhesive properties are thus activated on the endothelial surface so that circulating cells, including malignant cells, are able to adhere to these surfaces. The concept that cancer triggers an inflammatory reaction in the brain in response to injury is also pertinent to this issue (22). If this idea is correct, cell complexes composed of tumor cells and activated platelets can arrest in the brain microvasculature and induce the release of cytokines. This, in turn, can initiate an injury reaction, which can facilitate the entry of tumor cells into the brain. In addition, endothelial cell growth factors can regulate tight junction components, causing alterations in BBB permeability. The vascular endothelial growth factor, fibroblast growth factor, tumor necrosis factor, interleukins, and interferons are some of the proteins that are commonly associated with malignancies and that can affect the BBB. These observations highlight the fact that the BBB is an intricate and tightly regulated structure that can be influenced by various factors that may be involved in the malignant process. Of particular relevance, malignant cells have to adhere to and transgress the endothelium of the brain before they can establish a metastatic focus, making this event a critical step in the metastatic process.

The Brain Interstitium and Initial Growth of the Metastatic Focus. Metastatic brain lesions have a predilection to localize to the junction between the gray and white matter (11). This region of the brain coincides with the vascular border zone where blood vessels form whorls and loops that are believed to produce hemodynamic circumstances that favor the adhesion of metastatic cells. Although there is evidence of migration and invasion of tumor cells once the initial nidus is formed, radiologic and histopathologic data support the fact that most

metastatic lesions occur as spherical masses that grow locally at the initial site of tumor foci arrest in the brain. Recent studies also support the theory that tumor cells can adhere to the vascular endothelium and proliferate within the blood vessel, forming tumor masses even prior to entry into the parenchyma of target organs, including the brain (23). Following this event, the tumor cells physically disrupt the BBB, allowing cellular entry into the parenchyma where a larger tumor focus is established through secondary growth. Conjecturally, such a mechanism could also activate cell signaling pathways associated with injury and inflammation. This activated cascade induces degradation of local tissue and expression of molecules that promote the processes of migration of tumor cells into the brain substance, subsequent local invasion, and the initiation of angiogenesis. It is also known that tumor cells can interact with platelets, forming cellular aggregates that can adhere to the endothelium of capillaries (24, 25). If such aggregates induce regional ischemia is uncertain, but plausible. Metastatic lesions often present with high signal on diffusion-weighted MRI images, a fact that is attributed to edema and related "T2 shine-through." However, these regions could also potentially indicate the existence of small areas of local ischemia at the point where tumor cells lodge within the small capillaries. If aggregates such as these are able to enter the deep, penetrating branches of the cerebral circulation and induce ischemia, yet another mechanism is available for creating alterations in the BBB and producing a route of entry for tumor cells into the brain. Supporting this possibility, Doi et al. showed that experimentally induced ischemia can increase the number of metastatic lesions in the liver from colon cancer in association with an increase in E-selectin expression (26). Other studies using the same ischemia model have shown that tumor cells overexpressing Galectin-3, a β-galactoside binding protein, efficiently form metastatic liver lesions compared with control tumor cells (27). Although these mechanisms are theoretically plausible as influencing the formation of brain metastases, a survey of the literature reveals few studies directed towards explicating these putative mechanisms. Hence, their relevance to the establishment of brain metastases remains to be determined.

Once the metastatic cell traverses the endothelium, it enters the brain interstitium, a complex but poorly understood environment in which subsequent tumor growth occurs. Sulfated matrix proteoglycans, composed predominantly of heparan sulfate and to a lesser extent, chondroitin and dermatan sulfates, form a major constituent of the brain extracellular matrix (ECM). Proteoglycans intimately interact with and are subject to degradation by invading metastatic tumor cells. In vitro studies using brain metastatic melanoma cells and brain endothelial cells have shown that these two cell populations can cooperate in producing heparanase, a degradative enzyme which cleaves heparan sulfate, and in concert foster local break down of the architecture of the brain matrix (28). Similarly, astrocytes can interact with metastatic melanoma cells and induce heparanase production, again promoting matrix degradation (29). Marchetti et al. showed that the increased production of heparanase was mediated by the interaction between neurotrophins such as NGF and NT-3 produced by normal cells within the brain and the low affinity neurotrophin receptor, p75NTR, which is expressed by invading tumor cells (30). Other important matrix-degrading proteins such as metalloproteinases have also been strongly implicated as participating in local invasion of metastatic lesions. In a study by Okada, MMP-2 and MT1-MMP expression was localized to the tumor cells and gelatinolytic activity was seen within nests of metastatic carcinoma cells by in situ zymography, strongly suggesting a role for these processes in local degradation of the ECM (31).

In addition to remodeling of the brain ECM by invading metastatic cells, the establishment of the initial tumor focus requires the recruitment of autocrine and paracrine signals, including various growth factors, into the regional environment. Our knowledge of these events is mainly derived from studies of brain metastases from melanoma in which tumor cells were shown to elaborate various factors such as TGF-α, TGF-β, βFGF and IL-1β. These factors are postulated not only to keep tumor cells alive by autocrine and paracrine mechanisms but also to induce the production of heparanase, which

contributes to matrix degradation (32). It has also been shown that a paracrine form of transferrin may play a role in establishing brain metastases, particularly because brain-metastatic cells express high levels of transferrin receptors, which can bind low levels of transferrin in the brain parenchyma and initiate biologic changes such as increased invasion and proliferation (33). Given that in experimental models, most tumor cells extravasate but only a few cells are able to survive and establish larger tumor foci (34), factors that promote tumor survival become highly significant in the development of brain metastases.

5. THE RELEVANCE OF TUMOR SPECIFIC FACTORS

There is sufficient evidence to show that the process of metastasis occurs in distinct stages (Table 1), each of which presents a substantial barrier for the metastatic cell that it must sequentially overcome before establishing itself in the target tissue (35). The complexity of this process is marked by discrete hurdles that must be overcome by metastatic cells before they can survive and grow in the host tissue. This process highlights the important fact that cells that are destined to survive form a special subpopulation within the primary tumor that possesses intrinsic properties used to facilitate their survival (36). Accordingly, some tumors are believed to incorporate cells with intrinsic characteristics that allow them to metastasize to the brain, whereas others do not possess cells with these characteristics. Several elegant studies have shown that the metastatic process is governed at each step by pathologic molecular interactions. To establish a metastatic focus, these interactions mimic normally occurring physiologic contacts, resulting in the abnormal recruitment of molecular mediators that are normally involved in physiologic cell-to-cell interaction and that generate cell survival and proliferation signals. The degree of production and recruitment of such molecules is likely a defining characteristic of tumor cells with metastatic potential. By analyzing the rate-limiting steps in the various stages of metastases, several molecules have been identified that appear to be indispensable to tumor cells for establishing a remote malignant focus. The role of molecules necessary to promulgate metastasis may be conveniently considered in relation to the various stages of the metastatic process and may be categorized based on their normal physiologic functions in the body, such as adhesion, invasion, angiogenesis, and proliferation. The following sections outline the mediators of these molecular events that are critical for the metastatic process once a tumor cell has reached the brain.

Table 1. Brain Metastasis: Stages in development.

Stage	**Role of Host tissue**
Intravasation from primary site	
Transit via blood circulation	Adhesion to platelets
Host Tissue phase	
Adherence to brain endothelium	Facilitation of adhesion
Extravasation	Production of degradative enzymes
Primary growth phase	Neurotrophin interaction
Recruitment of blood supply	Response of brain vasculature
Secondary growth phase	Blood supply

6. MOLECULAR MEDIATORS OF METASTASIS

6.1 Mediators of Adhesion

Overview. A circulating tumor cell exhibits its organ specificity when it adheres to the endothelium of a target organ (37). Thus, the arrival of a tumor cell into the brain via the cerebral vasculature and its adherence to the vessel walls signals the first step of a direct interaction between the metastatic cell and the brain. This step is partly due to a *physical* arrest of the cell governed by mechanical and hemodynamic factors operant in the microvasculature of the target organ (38). Equally important in metastatic localization is the adherence of the metastatic cell to an endothelial cell via *molecular* interactions between the tumor cell and the subendothelial ECM (39). Continuous blood flow in the blood vessels of the central nervous system generates considerable shear forces and is a potent inhibitor of the adhesion of cells in the vascular component, including those derived from malignancies. To overcome these forces, a metastatic cell utilizes specific and robust molecular mechanisms involving adhesion molecules (Figure 2 A). In vivo studies using endothelial cell monolayers in mice demonstrated the specificity of interaction between tumor cells and the capillary endothelium. In this context it has been seen that tumor cells express cell adhesion molecules that are involved in normal physiologic adhesive interactions. Several such molecules have been implicated in the metastatic process, including intercellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1), epithelial cell adhesion molecule (EP-CAM) (40) and selectins, which constitute a family of cell-adhesion molecules including L-selectin/CD62L, E-selectin (CD62E), and P-selectin (CD62P) (41). These molecules are known to be involved in the interaction between cellular components of the blood stream such as leukocytes and the endothelial cells lining the vasculature, but have also attracted interest into the molecular mechanisms of the malignant process. Studies of these interactions not only provide a better understanding of tumor biology, but also are of particular interest from a therapeutic standpoint.

In addition to the interaction between tumor cells and the endothelium, recent reports provide evidence that some metastatic cells may overexpress cell surface integrin receptors such as $\alpha V\beta 3$ integrin that enables them to interact with integrins on platelet surfaces such as $\alpha IIb\beta 3$ (42). This association results in the formation of microthrombi that promote cell stasis in regions of slower blood flow, enabling the tumor cell to establish contact with mediators of adhesion on the endothelium of the target organ (Figure 2 B). The interaction between cell surface integrin receptors and activated platelets requires a functionally activated subtype of $\alpha V\beta 3$ integrin. The parental tumors in one study contained a subpopulation of cells which constitutively expressed activated $\alpha V\beta 3$ integrin, suggesting that parental cells may be primed for the metastatic process if they achieve anchorage independence from the primary tumor (43, 44). A similar interaction has been described between tumor cell surface heparin sulfate proteoglycans (HSPGs) and P-selectin on platelets, resulting in adhesion of platelets to the tumor cells (45). In addition, an increased serum concentration of VCAM-1 was shown to be associated with locally advanced metastatic gastric cancer (46). Patients with these advanced cancers also had a significantly poor survival compared with patients who had normal levels of these molecules.

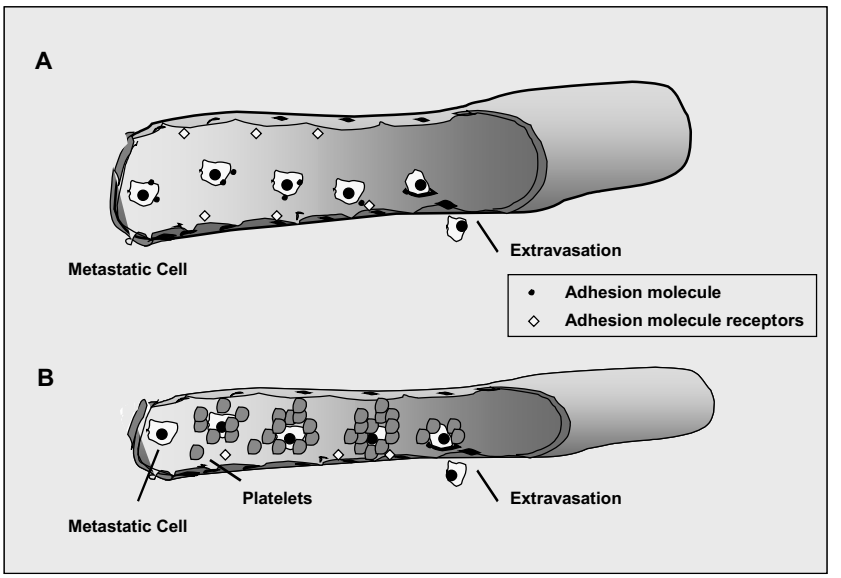

Figure 2. Mechanisms of adhesion of metastatic tumor cells to brain endothelium. A. Tumor cells express adhesion molecules on their surface, which interact with their respective receptors, B. tumor cells express molecules that promote adhesion to platelets and activate them to form microthrombi. The tumor-platelet complexes may lodge physically in the endothelium and the activated platelets may trigger changes in the blood-brain barrier that facilitate extravasation.

The specificity of interaction between tumor cells and specific organs such as the brain cannot be explained on the basis of general adhesion interactions alone because such interactions are likely to be physiologically active in several organs in a nonspecific way. As such, it is likely that molecules are expressed in the brain endothelium that specifically interact with adhesion molecules on the tumor cell surface and enable target tissue-specific adhesion of the cell as the first step in the process of metastasis. Whether such adhesion is required for metastasis to occur is controversial. A capacity for the adhesion and extravasation of tumor cells does not necessarily correlate with metastatic potential (47). However, it is clear that without adhesion and extravasation, even those cells destined to become metastatic will be unable to reach the target site. Adhesion interactions between tumor cells might, however, contribute to the overall load of tumor cells congregating in a specific metastatic site. Although the adhesion interactions between endothelial cells and tumor cells are not the sole regulators of metastasis, the interaction is an important first step that allows entry of metastatic cells into the brain.

Immunoglobulin-Like Cell Adhesion Molecules (CAMs). This family of adhesion molecules is characterized by their similarity to and evolutionary relationship with the immunoglobulin family (48). Several of these molecules have been postulated as playing a part in normal adhesion functions in the brain as well as in pathologic processes such as metastasis. Of these, the role of the neural cell adhesion molecule (NCAM) has been implicated in axonal growth and cell-cell interactions in the brain and the retina. NCAM is relevant to brain metastasis by virtue of the observation that it was constitutively expressed in melanoma cells isolated from brain metastatic lesions but not in tumors from other organs. Its expression in melanoma cells suggested that NCAM has a role as an immunoregulatory molecule during the formation of brain metastasis (49). NCAM is able to modulate metastasis by regulating tumor cell-matrix adhesion interactions; inducing FGFR-4 mediated signaling, which is responsible for

producing neurite outgrowth and matrix adhesion of tumor cells (50). Similarly, another adhesion molecule, intercellular cell adhesion molecule-1 (ICAM-1) is found to be selectively expressed in metastatic melanoma cells (not by other malignant cell types) but not in the primary lesion (51). Under physiologic conditions, ICAM-1 is expressed at low levels in the endothelial cells of the brain microvasculature (52) and the molecule plays a significant role in cell migration across the BBB, particularly leukocyte infiltration associated with inflammatory processes. Elevated levels of soluble ICAM-1 have been found in several malignancies and are related to development of angiogenesis (53). One report showed that the levels of ICAM-1 increased rapidly on the luminal surface of the endothelium when cell adhesion occurred and demonstrated an increased interaction with integrins as well as changes in protein phosphorylation and cytoskeletal reorganization Anti-CAM antibodies blocked the interaction between the tumor cell and endothelial cell. VEGF can upregulate ICAM-1 expression through the PI-3 kinase/AKT pathway (54). Blockage of the PI-3 kinase/Akt pathway with cell permeable inhibitors abolished this effect, suggesting that migratory events that might be controlled by this pathway, including interactions between metastatic cancer cells and the BBB, can potentially be disrupted. The role of CAMs in brain metastases is being further investigated and results of such studies will define the possibilities of therapeutically targeting these molecules.

Integrins. Integrins are a large family of cell surface adhesion receptors that interact with diverse intra- and extracellular stimuli to promote cell-cell interactions and related biological processes (55). They occur as heterodimers consisting of α and β subunits and exhibit a range of overlapping interactions with their ligands, which depend on the particular combination of subunits recruited (56). By virtue of their transmembrane position, they are capable of interacting externally with the ECM and internally with the cytoskeleton, thus providing a dynamic bridge for transmembrane communication between the cell and its environment. Upon interaction with ECM proteins via the Arg-Gly-Asp (RGD) motif, integrins cluster together at the point of contact and assemble cellular actin filaments, which results in the progressive, lateral recruitment of additional integrin molecules that combine to form the focal adhesions (56). In addition, integrins recruit several adaptor and signaling molecules, including focal adhesion kinase (FAK), src, Fyn, Talin, Vinculin and Paxillin (57). This activity results in the activation of the Ras, Rho and MAPK pathways, partly through the phosphatidyl inositol 3-kinase pathway, resulting in a spectrum of signals that can impact motility, cell cycle control, cell survival and proliferation (58-60). Of the integrin family members, the αv heterodimeric receptors form a distinct sub-family, which serve as vitronectin receptors (except $\alpha v\beta 6$), share the property of recognition of the RGD-motif in their ligands, and are implicated in malignancies (61). The most studied of these molecules is the $\alpha v\beta 3$ integrin, whose participation has been implicated in metastatic disease, and whose expression occurs in late stages of specific malignancies, including primary brain tumors (62). The $\alpha v\beta 3$ integrin interacts with various substrates, thus enabling tumor cells that express it to adhere to different substrates and interact with them in diverse environments (63). Cells overexpressing $\alpha v\beta 3$ integrins have an increased capacity to invade in Matrigel assays. Inducing the expression of this integrin in poorly invading cell lines increases their ability to invade. In addition, the interaction between $\alpha v\beta 3$ integrin and the ECM has been identified as an important factor for the survival of endothelial cells in newly formed blood vessels (64). VEGF-A can induce the expression and activation of $\alpha v\beta 3$ integrins, thus providing one mechanism whereby tumor cells might recruit a blood supply locally and ensure the integrity of newly formed blood vessels (65). Wang et al. (66) demonstrated that circulating tumor cells express $\alpha 3\beta 1$ integrins on their surface. These can interact with its ligand, LN-5, which in turn is expressed in areas of exposed basement membrane in the pulmonary vasculature, providing a molecular basis for occurrence of lung metastasis (66). Based on these data, small molecule inhibitors of integrins are currently in preclinical and early clinical testing against malignancies, including metastatic disease. One such agent is the cyclic RGD-motif peptide, Cilengitide (EMD121974),

which is a αvβ3 and αvβ5 integrin inhibitor (67). Cilengitide has recently completed phase I trials in humans and is entering phase II trials (68). Other agents of interest include Vitaxin, an anti-integrin humanized antibody that has entered clinical trials (69) and the RGD-peptidomimetic agents, S137 and S247, which can inhibit αvβ3, α5β1and α2β3integrins and decreased colon cancer metastasis in animal models (70).

Selectins. Selectins are a family of CAMs, which includes L-selectin (CD62L), E-selectin (CD62E) and P-selectin (CD62P), whose activities include mediating the capture of leukocytes from the blood stream as they reach the cerebrovascular endothelium. Selectins interact with vascular glycoproteins in the context of a carbohydrate structure called Sialyl Lewis x (SialylLex) (71). In the setting of metastasis, breast and lung carcinoma cells express glycoprotein molecules that can function as ligands for P-selectin such as P-selectin glycoprotein ligand-1 (PSGL-1), and CD24. Also SialylLex is abundantly expressed on the surface of epithelial malignant cells. Similarly, epithelial cell cancers express heparan sulfate-like proteoglycans, which can also function as ligands for P- and E-selectins, thus suggesting their role in metastasis (72). It has been suggested that these ligands interact with P-selectin that is expressed on circulating platelets, promoting the formation of a platelet aggregate around the tumor cell which not only protects it from the immune system but also facilitates impaction of the cells in small microcapillaries allowing adhesion to occur in the target tissue (44, 45, 73). Such selectins might also permit interaction between the tumor cells and an activated endothelium that expresses E- or P-selectins. The ability of heparin to inhibit metastasis in rodent models has been linked to its ability to inhibit P-selectin (74, 75). Several selectin inhibitors are currently in development against pathologic states other than cancer but are likely to be studied in the milieu of malignancies, especially metastases (76).

Tetraspanins. Tetraspanins constitute a superfamily of an evolutionarily conserved group of transmembrane proteins with four transmembrane domains and with surface domains that interact with various integrins and are implicated in the metastatic process (77, 78). α3β1 integrins can form complexes with tetraspanins that can control elongation of invading pseudopodia of tumor cells. These complexes have also been implicated in matrix metalloproteinase-2 (MMP-2) production, which is associated with tumor invasiveness (79). Tetraspanins act by modulating the actin cytoskeleton and assisting in degrading the surrounding ECM as the cancer cell advances through metastatic progression. By their prominent interactions with adhesion molecules such as integrins and with each other, they are involved in diverse processes such as cell activation and proliferation, adhesion and motility, differentiation, and tumorigenesis. However, their role in malignancy and metastasis is complex. Animal experiments have shown that expression of the tetraspanins CD9, CD63, or CD82 in tumor cells suppresses their metastatic potential (80, 81). In contrast, expression of CD151, which is expressed by cells with an epithelial and mesenchymal origin, increases invasion and the metastatic potential of tumor cells (82). CD151 forms stable complexes with the laminin-binding integrins α3β1, α6β1, α6β4 and α7β1 and can also associate with intracellular signaling molecules such as PKC-α and PKCβII, and the type II PI-4-kinase. The formation of such complexes may be required for the coordination of signals that regulate cell adhesion and migration. They may hence have a postulated role in brain metastasis, which remains to be defined. Lee et al. recently identified KITENIN, a novel tetraspanin, which when overexpressed, resulted in increased invasiveness and early metastasis (83). Given that tetraspanins are also widely expressed in the central nervous system, additional studies are warranted to determine the relevance of these molecules to brain metastases.

Focal adhesion kinase (FAK). FAK is a non-receptor tyrosine kinase and is detected in molecular complexes associated with focal contacts in the process of cell-cell adhesion. It is activated by tyrosine phosphorylation when ligands like vitronectin or other matrix proteins bind to integrin receptors, and is frequently associated with an

invasive phenotype (84). FAK binds to specific motifs in the cytoplasmic tail of β integrins and undergoes tyrosine phosphorylation, an interaction that is facilitated by a docking protein, Cas, which is itself activated by tyrosine phosphorylation (85). Recently, increased tyrosine phosphorylation of Cas has been shown to correlate with increased integrin-mediated cell migration in Cos cells (which are derived from the CV-1 cell line by transformation with a replication origin defective mutant of the SV40 virus). FAK is overexpressed in metastatic cells and is believed to contribute to the metastatic process by modulating invasion and motility (86). FAK is also expressed in cerebral metastases and has been found to interact with VEGF and nitric oxide signaling systems (87). VEGF increases the tyrosine phosphorylation of FAK and increases its localization to focal adhesions in endothelial cells (88), suggesting a complex interaction between angiogenesis signals and those that facilitate metastasis. In a recent study, Lu et al. reported that FAK is dephosphorylated in response to EGF treatment in human carcinomas that overexpress EGFR (89). This causes decreased activity of FAK, leading to the breakdown of focal adhesions and resulting in cells that become less adherent, more motile, and more prone to metastasis. However, following the re-adhesion of cells, FAK activity is restored via the integrin receptor pathway and the cells lose their sensitivity to EGF. This could provide a mechanism for intravasation, reattachment, and extravasation of metastatic cells.

6.2 Molecular mediators of Invasion and Angiogenesis

Overview. Once the tumor cell has adhered to the endothelium, it activates various mechanisms to enable it to traverse the endothelium and enter the brain parenchyma. Subsequently, after initial proliferation, the cells invade the brain parenchyma locally and activate angiogenic signals to form the metastatic focus. Although they are distinct biologic processes, invasion and angiogenesis share several common features and recruit the same molecular mediators. Molecules that participate in the process of angiogenesis include VEGF and its receptors, mediators of invasion such as matrix metalloproteinases (MMPs), urokinase plasminogen activator (uPA) and its receptor, and molecules involved in remodeling of ECM such as heparanase. A more detailed discussion regarding these molecules is presented in several reviews on this topic to which the reader is referred (90-93).

Matrix metalloproteinases (MMPs): Matrix metalloproteinases are a family of endopeptidases that predominantly exist in an inactive zymogen form and contain an active domain and a catalytic domain, the activity of which is zinc-dependent (91). They are elaborated by tumor cells in response to extracellular stimuli, including those from the ECM. Based on the ECM proteins that they preferentially degrade, MMPs are classified into three large groups – collagenases, stromelysins, and gelatinases (94). *Collagenases* are MMPs that act against several specific types of collagen, cleaving the proteins at defined sites into simpler products, which undergo further processing by other MMPs. S*tromelysins* form the second group of MMPs and are active in degrading various ECM substrates, including elastin, laminin, collagen and fibronectin. *Gelatinases,* the third class of MMPS, (also known as MMP-2 and MMP-9) are collagenases that have important roles in primary and metastatic brain tumor invasion, particularly because of their ability to induce degradation of the basement membrane (95).

Urokinase-like plasminogen activator (uPA) and receptor (uPAR). The uPA/uPAR system has a significant role in degrading the ECM by plasminogen activation at the cell-surface and is hence highly relevant to the malignant process especially with respect to invasion and angiogenesis. Upon binding to the uPAR, uPA initiates cleavage of plasminogen to plasmin focusing the proteolytic activity to regions of the cell that highly express the receptor such as the leading edge of migrating cells (96). This helps focal degradation of the ECM in specific locations aiding directional migration and invasion of the tumor cell. The uPA/uPAR system also cooperates with MMPs in inducing target tissue remodeling and permitting migration and invasion of metastatic cells. In addition to its extracellular tissue effects, αVβ3 integrin, the vitronectin receptor, and uPAR influence each other's expression and can

cooperate to induce adhesion and invasion (97). In glioma cells, downregulation of uPA resulted in reduced levels of phosphorylated PI3K and Akt, which are associated with decreased migration and invasion (98); this finding may also be relevant to brain metastatic cells. Similarly, down-regulation of uPAR expression in human colon carcinoma cells results in disrupted interactions with integrins and inhibiting the Erk-MAPK pathway (99). These events result in decreased invasion and migration of tumor cells, suggesting that uPAR participates in mediating an intracellular signaling pathway in invasion and, hence, metastasis. Thus, several lines of evidence suggest that the uPA/uPAR system is highly relevant to metastasis; structural analysis of the components of this system may allow targeted inhibition of this system as a therapeutic strategy against metastasis (100,101).

Heparanase and Heparan Sulfate Proteoglycans. HSPGs are important components of the endothelial basement membrane but also associate with the ECM and the cell surface. They are glycosaminoglycans composed of a core protein with multiple covalently linked heparan sulfate chains (102). The breakdown of HSPGs in the basement membrane is a critical step in the extravasation of tumor cell into the target organs. Heparanase is an endoglycosidase that degrades the heparin sulfate chains of HSPGs, thus breaching the basement membrane (BM) (103). Heparanase activity is normally seen in platelets, leukocytes, and placental trophoblasts but has also been described in melanoma, lymphoma, and prostate cancer (104). Recently, Marchetti et al. reported that astrocytes produce heparanase and potentiate the invasion of metastatic melanoma cells derived from brain metastasis (29). It is likely that heparanases and HSPGs also play an important role in brain metastasis from other cancers and in a similar manner.

Hypoxia-inducible factor 1 (HIF-1). HIF-1 is a basic-helix-loop-helix transcription factor that has essential roles in mammalian development and physiology. In its functional state, HIF-1 forms a heterodimer composed of HIF-1alpha and HIF-1beta subunits. The expression of HIF-1alpha is closely linked to cellular hypoxia and is regulated by tissue oxygen concentration (105). When the tissue growth reaches a state in which the cellular consumption of oxygen outstrips its supply, HIF-1 levels are upregulated. Under such hypoxic conditions, HIF-1-regulated genes, including those for VEGF, erythropoietin, and enzymes of the glycolytic cycles, are actively transcribed. This facilitates improved oxygen delivery or adaptation of the cell to hypoxic conditions. The hypoxic environment in growing metastatic tumors induces the overexpression of HIF-1alpha, which activates adaptive mechanisms in the tumor and induces angiogenesis (106). Interestingly, HIF-1 is degraded by a mdm2-mediated mechanism, which is regulated by p35. Loss of p53 (commonly seen in malignancies) results in the inability of the cell to degrade HIF-1 through mdm2 (43). HIF-1 is essential for neovascularization in several metastatic cancers and plays an important role in tumor growth and survival.

Vascular Endothelial Growth Factor (VEGF). VEGF has been of particular interest in regard to BBB functions and, by extension, to the metastatic process. In a mouse model of brain metastasis from breast cancer, Kim et al. isolated a population of cells from brain metastatic lesions, which demonstrated an increased propensity for metastasizing to this organ (107). They observed that these cells secreted high levels of VEGF and that chemical inhibitors of VEGF caused a decrease in brain metastasis. The authors concluded that high VEGF levels contribute to the development of brain metastasis by tumor cells. VEGF also induces reorganization of vascular endothelial cadherin, an effect that is antagonized by the inhibition of PKC, Erk or eNOS. VEGF also upregulates levels of ICAM-1 and the chemokine MIP-1 α in association with decreased association between astrocytic foot processes and the vascular endothelium, thus weakening the integrity of the BBB and increasing vascular permeability (108). Recent studies showed that the permeability of the BBB is increased in response to VEGF, a relationship that is mediated by eNOS (109). Relevant to these findings is the observation by Martinez-Estrada et al. that systemically administered erythropoetin can protect

against VEGF-induced increased permeability of the BBB by reducing the levels of eNOS and restoring the structural integrity of the tight junctions (110). Approaches similar to this may potentially provide a mechanism to inhibit the adhesion of metastatic cells to the brain endothelium, thus preventing development of brain metastases.

Chemokines and their receptors. Tumor cell metastasis shares many characteristics of leukocyte trafficking in response to inflammatory and injury-related signals. Among the molecules involved in this process, chemokines have emerged as key mediators of cell-cell interactions, which also appear to play a role in metastasis. Chemokines are a family of small, secreted molecules that function significantly in leukocyte trafficking, particularly in response to injury and inflammation, and also function as ligands to a set of chemokine receptors. They are divided into several families on the basis of their specific structures and the cysteine residue-motifs in their peptide sequence as well as the specific receptors that they engage. Chemokine receptors are seven-transmembrane domain proteins belonging to the superfamily of G protein-coupled receptors, which are highly expressed on migrating cells. Upon ligand binding, the receptors signal integrins via protein kinase C and activate migration by modulating cytoskeletal components. Of the various families of chemokines the CXC family has been specifically associated with metastases and angiogenesis. In breast cancer, SDF-1 (CXCL12), which serves as a ligand to the chemokine receptor CXCR4, is overexpressed compared with its expression in normal breast tissue (111). In vitro studies using breast cancer cells showed that SDF-1 stimulation caused PI3K activation, promoting survival signals and increased vascular permeability accompanying vascular instability. Interestingly, SDF-1-stimulated cells also showed increased migration and ability to penetrate brain microvascular endothelial cells; treatment with CXCR4-inhibiting antibodies or PI3K inhibitors abrogated this effect, suggesting a role for this chemokine receptor and the PI3K pathway in brain metastasis from breast cancer. Small molecule inhibitors against CXCR4 are currently in development in the hope that they will have the potential to be used as therapeutic agents against brain metastasis from breast cancer (112).

7. MOLECULAR PROFILING OF THE PRIMARY TUMORS AND ITS RELEVANCE TO METASTASIS

The theory that clonal selection is an underlying mechanism of tumorigenesis as well as of the evolution of tumor heterogeneity is now well accepted. Prevalent concepts that are thought to be relevant to metastasis propose that metastasis represents an overall process of genetic selection in which cells that eventually metastasize evolve during the later stages of the malignant process in a highly selective process (113). More recent evidence has suggested that the primary malignancy may contain cells that have the potential to metastasize to specific organs because of their inherent biologic characteristics rather than genetic selection. Presumably, such biologic characteristics would have to be predestined early in the evolution of the tumor so that biologic characteristics established in specific cells are triggered and allow the cells to interact with and survive in the target organ when metastasis occurs. Clearly, several other factors likely determine if these cells eventually reach target organs, including survival through primary therapies, detachment from the primary tumor focus, and ability to traverse the vascular compartment. It is important to emphasize that of the cells that are released from the primary malignancy and reach the brain, only those endowed with specific biologic characteristics can form metastases in that organ. Accordingly, it has been postulated that profiling the tumor as it exists in the primary site, either at diagnosis or at recurrence, could potentially prognosticate the potential of a given tumor to form brain metastases.

Comparing the molecular profiles of matched primary with brain-metastatic tumor tissue might reveal “signatures” in the latter that can provide clues to biologic characteristics that determine metastatic behavior. Weigelt et al. showed that the gene expression profiles of matched samples from primary breast cancer and metastatic lesions from

the breast, even if these were spawned or became apparent later in the course of the disease, were similar. The authors contended that this finding supported the concept that an inherent capacity to metastasize is a driving force behind metastasis rather than metastasis reflecting a process of individual genetic selection (114). Interestingly, these authors found that differences in microenvironment did not appear to affect this tissue similarity, pointing to a primary characteristic inherent in the tumor cell that causes it to metastasize and grow in a distant organ. The authors did not address the possibility that the cells could have metastasized early, remained dormant until favorable circumstances arose and thus established secondary foci later in the disease course. In another interesting study using infrared DNA spectra, Malins et al. compared the DNA base and backbone structure of histologically normal prostate tissue with matched prostate cancer tissue from patients with and without metastatic disease (115). Based on similarities between the DNA structure of histologically normal tissue and metastasizing primary tumor in matched samples, they suggested that the metastatic and primary phenotypes evolve independently, again suggesting the early emergence of cells with metastatic characteristics. They also found that histologically normal tissue from patients with metastasizing tumor had similar DNA structures and proposed that the metastatic potential was in progenitor cells, with metastatic features "hardwired" into the DNA.

Other investigators also demonstrated that cells that metastasized to specific organs bear characteristics that facilitate their localization to those specific organs and that these physiologic traits are distinct from the non-metastatic components of the primary tissue. In breast cancer cells metastatic to the brain (but not in cells from primary tissue), Nishizuka et al. found that several cytokine receptors were upregulated that could respond to astrocyte-derived cytokines and hypothesized that the metastatic cells would thus be better suited to respond to paracrine signals from the brain microenvironment (116). A similar role has been suggested for neurotrophins expressed by metastatic cells in promoting invasion and responding to astrocyte-derived signals in the brain by autocrine and paracrine mechanisms (30). It should be recognized that some of the differences in profiling studies could be due to the effect of the brain microenvironment on the tumor cell and not due to intrinsic properties of the tumor cell. Thus, it would be equally important to identify molecular features in subpopulations of the primary tumor cells that are "destined" to metastasize; such features would also be present in the metastatic cells at all stages of the metastatic process. Target organ-specific features present in metastatic cells would be absent in those primary tumors and metastatic cells that do not metastasize to the brain. Thus, comparative profiling between tumors that metastasize to the brain or those that fail to do so may help identify early molecular signatures that could guide patient selection as well as subsequent treatment. Significant efforts are currently ongoing to systematically study the biologic profile and molecular alterations of brain metastasis that potentially dictate their clinical behavior. (22, 117-119)

8. PREVENTION OF BRAIN METASTASIS BY MODIFICATION OF BIOLOGIC FACTORS – FROM BENCH TO BEDSIDE

A better understanding of the multistep process of brain metastasis will allow the identification of rate limiting steps in this disease that may permit therapeutic intervention. For malignancies that manifest with brain metastases early in the course of the disease, such as lung cancer, primary prevention of brain involvement may be challenging because these cancers may often present with brain lesions. In such cases, inhibition of angiogenesis, invasion, and disruption of signals that arise from the brain microenvironment to facilitate tumor growth in association with treatment of the primary malignancies could be a reasonable strategy. Identification of biologic 'Achilles heels' common to both the primary and metastatic lesions may facilitate using the same agent or combination of agents to treat the disease in its different locations. The limitations of drug delivery to the brain and

variations in tissue pharmacology between the primary site and the metastatic lesions could, however, potentially heighten the challenge of this approach. Combining such techniques with radiation therapy (stereotactic or whole brain) could allow the dose of radiation used in radiation therapy to be reduced, reducing the risk of toxicity, while increasing targeted activity against the brain metastases.

Malignancies that are associated with brain metastases late in the course of the disease will likely afford a better opportunity for primary prevention than earlier-occurring lesions. In such situations, clinical experience suggests that successful therapy of the primary disease does not ensure prevention of brain metastases, which may nevertheless appear later in the disease course in the absence of activity at the primary site. Preventing metastases from occurring in this setting would presumably require continuous suppression of a combination of factors responsible not only for brain metastases, but also for other systemic metastases along with treatment of the primary disease. Hence, identification of biologic factors that are universally common to metastases (such as those described in the sections above) but that are not involved in normal physiologic processes in adults may afford the best opportunity for this chemoprevention strategy. When brain metastases occur in the face of widespread metastases and a high tumor burden, treatments that target biologic characteristics common to the entire disease process or those that impact the components of the disease process that are most relevant to patient prognosis may be appropriate targets for intervention.

If it is true that a subpopulation of cells in the primary tumor is destined to metastasize and that the other cells do not evolve into such a metastatic phenotype, treatments that can target and eliminate such cells in the primary tumor early in the course of the disease may eliminate the possibility of metastases and obviate the need for chronic therapy. These approaches require a precise and comprehensive understanding of the molecular factors that determine biologic characteristics in the primary and metastatic tumors. In this context, ongoing studies using preclinical models, translational approaches and comprehensive profiling of primary and metastatic tissue will undoubtedly provide the basis for rational therapeutic approaches; in addition, active collaboration between industry, academia and government will be needed to focus attention on metastatic disease process as a priority area in the fight against cancer.

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