Melanoma Metastasis

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Cancer metastasis requires a series of sequential steps, each of which is rate limiting. Neoplasms are biologically heterogeneous, and the process of metastasis is highly selective. Multiple metastases often differ in biologic characteristics and can change during the course of the disease. Clonal analysis of human melanoma suggest that systemic, physiologic signals can be recognized by neoplastic cells. Brain metastases are particularly common in patients with metastatic melanoma. The blood brain barrier does not prevent the invasion of the brain parenchyma by circulating metastatic cells, and its permeability varies among different experimental brain metastases.

Introduction

When a diagnosis of melanoma is established, the most critical question is whether the cancer is localized or has spread to regional lymph nodes and distant organs. Most deaths from melanoma are due to metastases that are resistant to conventional therapies.[1] A major obstacle to therapy of metastases is tumor-cell biologic heterogeneity. By the time a diagnosis is established, melanomas contain multiple cell populations characterized by diverse growth rates, karyotypes, cell-surface properties, antigenicity, immunogenicity, invasion, metastasis, and sensitivity to cytotoxic drugs or biologic agents.[2,3] Moreover, in many patients with malignant melanoma, metastasis already may have occurred.[4] The metastases can be located in various lymph nodes and distant organs where specific microenvironmental factors can modify the response of melanoma cells to systemic therapy.[1]

An understanding of the mechanisms that regulate the pathogenesis of metastasis will lead to the ability to design more effective therapy for malignant melanoma. This review discusses basic concepts of cancer metastasis of malignant melanoma and describes how the host's environment influences the biologic behavior of metastatic cells.

Formation of Melanoma Metastasis

The process of cancer metastasis consists of a long series of sequential, interrelated steps (Figure), each of which is rate limiting. After the initial transforming event, growth of neoplastic cells must be progressive. Extensive vascularization (angiogenesis) must occur if a tumor mass is to exceed 2 mm in diameter.[5] The synthesis and secretion of several angiogenic factors play a key role in establishing a capillary network from the surrounding host tissue.[6] Invasion of the host stroma by some tumor cells occurs by several nonmutually exclusive mechanisms.[7] Thin-walled venules, like lymphatic channels, are easily penetrated by tumor cells and provide the most common pathways for tumor cell entry into the circulation. Detachment and embolization of small tumor-cell aggregates occur next, and the vast majority of circulating tumor cells are destroyed rapidly. Tumor cells that survive the circulation must be trapped in the capillary beds of organs. Extravasation follows next, probably by the same mechanisms that influence initial invasion, and development of vascularization and proliferation within the organ parenchyma completes the metastatic process. Tumor cells can invade host stroma, penetrate blood vessels, and enter the circulation to produce additional metastases, the so-called "metastasis of metastases." The outcome of the process depends on the interaction between the intrinsic properties of the tumor cells and the various host factors, which can vary among different patients.

Algorithm depicting the pathogenesis of melanoma metastasis. The process of metastasis is sequential and requires the completion of several highly selective steps.

Lymphatic Metastasis

Early clinical observations led to the impression that carcinomas spread mainly by the lymphatic route, while mesenchymal tumors such as melanoma spread mainly through the bloodstream. Since both the lymphatic and vascular systems have numerous connections that allow disseminating tumor cells to pass rapidly from one system to the other,[8] this concept is invalid. During invasion, tumor cells can easily penetrate small lymphatic vessels easily and be transported in the lymph. Tumor emboli may be trapped in the first draining lymph node, or they may bypass these regional lymph nodes (RLNs) to form distant nodal metastases ("skip metastasis"). Although this phenomenon was recognized in the late 1800s,[9] its implications for treatment were frequently ignored in the development of surgery for cancer.
In draining a primary neoplasm, RLNs may enlarge as a result of hyperplasia or growth of tumor cells in the nodes. The question of whether these lymph nodes can retain tumor cells and serve as a temporary barrier to cell dissemination is controversial. In most experimental animal systems used to investigate this question, normal lymph nodes were subjected to a sudden challenge with a large number of tumor cells, a situation that may not be analogous to that of RLNs at the early stages of melanoma spread in human beings, when small numbers of cancer cells continuously enter the lymphatics. This issue is important because of the practical ramifications for surgical management of cutaneous melanoma.[4] For example, the biologic justification for elective lymph node dissection in patients with melanoma presumes that metastasis of some cutaneous melanomas occurs first in the draining lymph node (sentinel lymph node) and tumor cells later gain access to the circulation to reach distant organs. If this is the case, resection of RLNs that contain tumor cells should increase the cure rate in subgroups of patients with melanoma. There is evidence to suggest that patients with melanomas of intermediate thickness (1 to 4 mm) have an improved survival rate after elective lymph node dissection.[4]

Hematogenous Metastasis

In the clonal origin of melanoma metastases, melano- 
mas must survive in order to adhere to small blood vessels or capillaries and then invade the vessel wall. The presence of tumor cells in the circulation does not constitute metastasis, since most circulating cells die rapidly. For example, using radiolabeled mouse B16 melanoma cells, we found that less than 0.1% of these tumor cells survived to produce metastases.[10] As the number of cells released by a primary tumor increases, the probability that some cells will survive to form metastases also increases. The number of tumor emboli in the circulation appears to correlate with the size and clinical duration of the primary tumor[11] and with the development of necrotic and hemorrhagic areas in large tumors. The rapid death of most circulating tumor cells probably is due to many tumor cell properties, such as increased adhesiveness due to altered expression of adhesive molecules. Host factors (e.g., blood turbulence, platelets, T cells, natural killer cells, and macrophages) also contribute to the death of blood-borne tumor emboli.[11] Moreover, the passage of tumor cells through capillaries leads to cell lysis by shear forces and by nitric oxide produced by cytokine-activated endothelial cells.[12] Once metastatic emboli reach capillary beds, they can become lodged to tumor cells and small blood vessel endothelial cells and then form stable adhesions between these cells. The survival and growth of metastatic cells at secondary organs also involve the responses by the cells to specific organ factors. Tumor cells can recognize tissue-specific motility factors that direct their movement and invasion. Following invasion of organ parenchyma, tumor cells also must respond to organ-specific factors that influence their growth.[13]

The fact that only a small number of cells in a primary tumor can produce metastasis led to the question of whether the development of metastases is due to random survival of few tumor cells or whether it represents the selective growth of unique subpopulations of tumor cells endowed with special properties. The majority of recent data conclude that neoplasms are biologically heterogeneous and that the process of metastasis is highly selective.

"Seed and Soil" Principle

Clinical observations of cancer patients and studies with experimental rodent tumors have led to the conclusion that certain tumors produce metastasis to specific organs independent of vascular anatomy, the rate of blood flow, or the number of tumor cells delivered to each organ. The distribution and fate of hemato- 

geneously disseminated, radiolabeled melanoma cells in experimental rodent systems demonstrate that tumor cells reach the microvasculature of many organs.[1] Proliferation of tumor cells, however, occurs only in some organs.

The search for the mechanisms that regulate the pattern of metastasis began in 1889 when Stephen Paget, MD, FRCS, analyzed 735 autopsy records of women with breast cancer.[9] The nonrandom pattern of visceral metastases suggested that the process 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 matched.[9]

Experimental data supporting the "seed and soil" hypothesis were derived from studies on the preferential invasion and growth of B16 melanoma metastases in specific organs.[14] The B16 melanoma cells were injected into the circulation of syngeneic mice. Tumor growths developed in the lungs and in fragments of lung or ovarian tissue implanted intramuscularly, but they did not develop in renal tissue implanted as a control or at the site of surgical trauma.[12] These results confirmed that sites of metastasis are determined by both the tumor cells and the specific microenvironment of the host tissue.

Although experimental analysis of cancer metastasis is performed in laboratory animals, ethical considerations preclude this analysis in human patients. However, the introduction of peritoneovenous shunt for palliation of malignant ascites has provided an opportunity to study some of the factors affecting metastatic spread in human patients.[15] Good palliation with minimal complications was reported for 29 patients with ovarian cancer or prostate cancer. The autopsy findings in 15 patients substantiated the clinical observations that the shunts did not increase the incidence of visceral organ metastasis. In fact, despite continuous entry of hundreds of millions of tumor cells into the circulation, metastases in the lung (the first capillary bed encountered) were rare, implying that circulatory anatomy per se does not determine the production of metastases.[15]

Metastatic Heterogeneity

Populations of cells that differ from the parent neoplasm in metastatic capacity can be isolated, thus supporting the hypothesis that not all the cells in a primary tumor can disseminate successfully. Two general approaches have been applied. In the first approach, metastatic cells are selected in vivo: tumor cells are implanted into mice and metastatic lesions are harvested. The cells that are recovered can be expanded in culture or used immediately to repeat the process. The cycle can be repeated, and the behavior of the cells is compared with that of the cells of the parent tumor. This procedure was originally used to isolate the highly metastatic B16-F10 line from the wild-type B16 melanoma[16] and it also has been used successfully to produce tumor cell lines with increased metastatic capacity from many other experimental tumors.[1] In the second approach, cells are selected for the enhanced expression of a phenotype believed to be important in one or another step of the metastatic sequence, and then they are tested in the appropriate host to determine whether concomitant metastatic potential has been increased or decreased.[1]

In 1977, Margaret Kripke and I provided the first experimental proof for metastatic heterogeneity in neoplasms in work with the mouse B16 melanoma.[17] Using the modified fluctuation assay of Luria and Delbruck, we showed that different tumor cell clones, each derived from individual cells isolated from the parent tumor, varied dramatically in their ability to form pulmonary nodules following intravenous inoculation into syngeneic mice. Control subcloning procedures demonstrated that the observed diversity was not a consequence of the cloning procedure.

To exclude the possibility that the metastatic heterogeneity found in the B16 melanoma might have been introduced as a result of lengthy cultivation, we studied the biologic and metastatic heterogeneity in a mouse melanoma induced in C3H/HeN mice by chronic exposure to ultraviolet B radiation and painting with croton oil. One mouse thus treated developed a melanoma designated K-1735. The original K-1735 melanoma was established in culture and immediately cloned. The clones differed greatly from each other and from the parent tumor in their ability to produce lung metastases. Moreover, the metastases varied significantly in size and pigmentation. Metastases to the lymph nodes, brain, heart, liver, and skin were found in addition to lung metastases.[18]

Metastasis of Metastases

We then examined whether cells that populate metastases possess a greater metastatic capacity than most cells in the parent neoplasm.[19] Some support for this possibility comes from the initial in vivo selection experiments on the highly metastatic B16-F10 cell line derived from the parent B16 melanoma. Comparable results have now been obtained with the K-1735 tumor. When cells from the parent tumor were injected intramuscularly into the hind footpads of syngeneic mice, the resulting skin tumors produced spontaneous pulmonary metastases. Four cell lines that were established from four individual lung metastases harvested from four different mice produced significantly more metastases than cells of the parent line, thus providing additional evidence for the hypothesis that metastasis is a selective process.[19]

If cells populating metastases have increased metastatic properties, then metastasis from a metastasis is likely to occur. To explore this possibility, we injected metastatic B16 cells into the hind footpads of syngeneic mice. When a tumor reached 12 mm in diameter, the affected leg was amputated at the mid femur. All such mice were then examined para- biotically to normal syngeneic mice. Three weeks later (after common circulation was established), the parabiotic animals were separated. Several weeks later, all mice developed lung metastases. The mice injected with melanoma cells in the footpad developed metastases from the local "primary" tumor, and the parabiotic mice developed metastases from the lung metastases.[20]
Generation of Biologic Diversity Within a Metastasis

Clinical and histologic observations of neoplasms have suggested that tumors undergo a series of changes during the course of the disease. For example, a tumor initially diagnosed as benign can evolve over a period of many months into a malignant tumor. This evolution can be demonstrated by the case of human cutaneous melanoma, in which the transformation of normal melanocytes and their conversion into metastatic cells have been studied in detail.[22] This gradual progression consists of a series of discrete and irreversible steps. To explain the process of tumor evolution and progression as originally defined in 1954,[23] Nowell[24] suggested that acquired genetic variability within developing clones of tumors, coupled with host-selective pressures, can result in the emergence of new tumor-cell variants that exhibit increasing growth autonomy or malignancy. Nowell's hypothesis predicted that accelerating tumor progression toward malignancy is accompanied by increasing genetic instability of the evolving cells. To test this hypothesis, we examined the metastatic stability and rates of mutation of primary metastatic and nonmetastatic cloned lines isolated from four different mouse neoplasms and found that highly metastatic cells were phenotypically less stable than their nonmetastatic counterparts. Moreover, in highly metastatic clones, the rate of spontaneous mutation was several times that of low metastatic clones.[25] These results are in accord with the hypothesis that tumor progression occurs as a result of acquired genetic alterations. Similar data have been reported for other neoplasms.

Melanoma Brain Metastases

Cerebral metastases are clinically diagnosed in 40% to 60% of patients with metastatic melanoma, an incidence that increases to 70% to 90% at autopsy.[26] We recently have described the development of a mouse model with which to study cerebral metastasis after injecting syngeneic tumor cells into the internal carotid artery. This technique simulates the hematogenous spread of tumor emboli to the brain and examines the final steps of the metastatic process: release of tumor cells into the circulation, arrest of tumor cells in capillaries, penetration and extravasation of the tumor cells into the brain through the blood-brain barrier, and continuous growth of the cells in the brain tissue.[27] A remarkable difference was found between two murine melanomas in patterns of brain metastasis: one melanoma produced lesions only in the brain parenchyma, whereas the second produced growths in the meninges and ventricle.[28] The same technique was used in nude mice to evaluate the biologic behavior of cells from eight different human melanomas. Direct intracranial injection of tumor cells demonstrated that all eight human melanomas were capable of growing in the brain of nude mice, and all but one human melanoma produced experimental brain metastasis (tumor lesions) following intracardiac injection. These metastases were found in the meninges, ventricles, and parenchyma, and each melanoma showed a slightly different pattern of growth in different regions of the brain. However, cell lines derived from two different primary human melanomas in 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 nude mice.[29]

Site-Specific Brain Metastasis

To determine the mechanisms that regulate site-specific brain metastasis, we transfected melanoma cells with DNA from plasmids pSV2neo or pSV2hygro, which confer resistance to the drugs neomycin and hygromycin, respectively. Hybrids between the B16 and K-1735 cells were obtained by fusion. Cells of the K-1735 x K-1735 hybrid grew in the brain parenchyma of C57BL6 x C3H(Hen) F1 mice, whereas all B16 x K-1735 hybrids produced lesions only in the meninges and the ventricles.[30] Theoretically, the differences in site-specific brain metastasis observed among the different melanomas could be due to different behaviors at different steps of the metastatic process. Following intracardiac injection, tumor cells must first reach the microvasculature of the brain, arrest, extravasate into the organ parenchyma, and then proliferate into measurable lesions. Using the two parental melanomas and several somatic cell hybrids, we searched for differences in cell arrest, extravasation, and growth that could account for the presence or absence of brain parenchymal melanoma lesions.[30]

The arrest of cells in the capillary bed is regulated by multiple factors that include adhesion molecules and the size of circulating emboli. The expression of cell-surface CD44 has been shown to play a role in organ-specific homing of lymphocytes. This molecule binds to components of the extracellular matrix such as fibronectin, hyaluronate, and collagen types I and IV. Recent reports have correlated the expression of CD44 on mouse or human melanoma cells with metastatic potential.[31] In our study, neither expression of cell-surface CD44 nor formation of homotypic aggregates correlated with initial cell arrest, as measured by the survival of 125I-labeled iododeoxyuridine cells with the site of tumor growth. As previously shown, initial cell arrest in brain parenchyma or meninges did not presage the eventual growth of cells into metastases.[30]

Once cells are arrested in a capillary bed, they can extravasate into the organ parenchyma. Increased cell motility and production of degradative enzymes facilitate this process. On the other hand, the production of degradative enzymes by tumor cells in capillaries, penetration and extravasation of the tumor cells into the brain through the blood-brain barrier, and continuous growth of the cells in the brain tissue.[27] The production of gelatinases by BL-4H cells showed invasiveness. The production of gelatinases by BL-6N/C-4H hybrid cells was inhibited by TGF beta (TGF-beta), but not by the K-1735/C-4 cells. However, since both the BL-6N/C-4H hybrid cells grew rapidly on the leptomeninges, the brain parenchyma was not infiltrated by the BL-6N/C-4H hybrid cells. The brain parenchyma was not infiltrated by the BL-6N/C-4H hybrid cells, whereas it was infiltrated by the K-1735/C-4 and C-4H/C-4 cells, and some invasion by the BL-6N/C-4H cells was seen. The cells invaded via the Virchow-Robin space surrounding blood vessels and directly connected to the subarachnoid space. Since the B16 and BL-6N/C-4H cells did not proliferate in the brain parenchyma (except after direct intracerebral injection), we concluded that either the absence of stimulatory growth factors or the presence of inhibitory growth factors in the microenvironment could account for this finding.[30]

We cultured the various melanomas in vitro in the presence of several growth factors previously shown to influence growth. The presence of epidermal growth factor (EGF), basic fibroblast growth factor (FGF), or platelet-derived growth factor (PDGF) did not influence growth of any of the cell lines tested. Significant differences were found when the cells were cultured with transforming growth factor-beta (TGF-beta). TGF-beta is a highly conserved, homodimeric, receptor-mediated, 25-kDa multifunctional regulatory protein. In addition to its effects on cell proliferation and differentiation, TGF-beta regulates many biologic processes such as glycosylation, angiogenesis, extracellular matrix metabolism, protein phosphorylation, and liver regeneration. More important, TGF-beta is functionally interactive with many hormones and growth factors such as EGF, PDGF, and FGF, suggesting a signal-transducing role for TGF-beta.

In contrast with the inhibitory effects on the B16BL-6N and BL-6N/C-4H hybrid lines, the lack of effect by TGF-beta on K-1735/C-4 cells and the stimulation of C-4H/C-4 hybrid cells may explain at least in part the differential parenchymal and meningeal growth patterns of these cells in the brain environment. We found that the divergent effects of TGF-beta correlate with the difference in the apparent binding of TGF-beta to these cells. Both TGF-beta1 and TGF-beta2 stimulated the growth of K-1735/C-4 and C-4H/C-4 hybrid cells. The growth of both B16BL-6 and BL-6N/C-4H hybrid cells was inhibited significantly by TGF-beta1 or TGF-beta2. Since TGF-beta2 is highly concentrated in the brain, our findings suggest that the failure of B16BL-6 or BL-6N/C-4H hybrid cells to produce intraparenchymal brain lesions could be due to their sensitivity to growth inhibition by TGF-beta.[30]

Blood-Brain Barrier and Melanoma Metastasis

The microvasculature of the brain parenchyma is lined with a continuous, nonfenestrated endothelium with tight junctions and little pinocytic vesicle activity. This structure, designated the blood-brain barrier, limits the entrance of circulating macromolecules into the brain parenchyma. The blood-brain barrier and the lack of a lymphatic system...
maintain the brain as an immunologically privileged site and protect the brain against both the entry of most drugs and the invasion by microorganisms. However, the blood-brain barrier does not prevent the invasion of the brain parenchyma by circulating metastatic cells. Melanoma cells can traverse a barrier in the brain that otherwise prevents the entry of most circulating macromolecules and microorganisms. In contrast to the brain parenchyma, capillaries in the meninges and the choroid plexus are lined by a fenestrated endothelium, which presents a lesser physical barrier to invading tumor cells.[32] We found that human melanomas that formed the most extensive parenchymal metases in the brain of nude mice were originally isolated from brain parenchymal metastases. In comparison, human melanoma lines derived from extracerebral metases produced lesions more frequently in the meninges or choroid plexus. One possibility for this difference is that the lines derived from brain metasteses already had been selected (in the patient) for their ability to cross the blood-brain barrier and grow in brain parenchyma. This pattern of metastasis is determined by interactions between the metastatic cells and the organ environment, possibly in terms of response to local growth factors or inhibitors. Of several molecular tracers used to study the permeability of the blood-brain barrier, we chose sodium fluorescein. Despite its low molecular weight (Mr 376), this hydrosoluble molecule is excluded from the brain by an intact blood-brain barrier. 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.[33] Before studying the function of the blood-brain barrier in such brain lesions, we ruled out the possibility 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 damaged the endothelial cells of the cerebromicrovessels and thus changed the permeability of the blood-brain barrier. Histologic examination revealed two patterns of tumor growth. In the first pattern, tumor cells formed isolated, well-defined nodules in the parenchyma of the brain. The blood-brain barrier was intact in lesions smaller than 0.2 mm squared until the small tumor-cell colonies coalesced to form large tumor masses.

The integrity of the barrier around small lesions (metastases) shows that the barrier is intact after passage of metastatic cells into the brain parenchyma. Moreover, the interaction of astrocytes with endothelial cells and elongated cytoplasmic processes of oligodendrocytes is likely to be important in maintaining a functional blood-brain barrier. 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 metastases might be responsible for the normal function of the blood-brain barrier. Because the blood-brain barrier is not intact in experimental brain metastases that exceed 0.2 mm squared, the resistance to chemotherapy may be due to other mechanisms. These results suggest that the permeability of the blood-brain barrier varies among different experimental brain metastases and that its function is related to the growth pattern and size of the lesions.[33]

Conclusions

A series of linked, sequential steps must be completed by tumor cells if a metastasis is to develop. Although some of the steps in this process contain stochastic elements, metastasis as a whole favors the survival and growth of a few subpopulations of cells that preexist within the parent neoplasm. Moreover, metastases can have a clonal origin, and different metastases can originate from one proliferation of single cells. The outcome of metastasis depends on the interaction of metastatic cells with different organ environments. Organ-specific metastases have been demonstrated in a variety of experimental tumor systems, and tumor growth has been found that is specific to a particular site within one organ.

Studies have shown that the implantation of human cancer cells derived from surgical specimens into correct anatomical sites of nude mice provides a suitable model of metastasis of human tumors. Clonal analysis of a human melanoma has revealed that these tumors are heterogeneous for metastatic properties and that growth in the environment of specific organs can be selective. These data suggest that systemic physiologic signals can be recognized by neoplastic cells presumably by mechanisms similar to those shared by their normal cell counterparts. Elucidation of the mechanisms that regulate metastasis will lead to better therapeutic interventions.

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