

Tumor Cell-Organ Microenvironment Interactions in the Pathogenesis of Cancer Metastasis

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The process of cancer metastasis is sequential and selective and contains stochastic elements. The growth of metastases represents the endpoint of many lethal events that few tumor cells can survive. Primary tumors consist of multiple subpopulations of cells with heterogeneous metastatic properties, and the outcome of metastasis depends on the interplay of tumor cells with various host factors. The findings that different metastases can originate from different progenitor cells account for the biological diversity that exists among various metastases. Even within a solitary metastasis of proven clonal origin, however, heterogeneity of biological characteristics can develop rapidly.

The pathogenesis of metastasis depends on multiple inter-

actions of metastatic cells with favorable host homeostatic mechanisms. Interruption of one or more of these interactions can lead to the inhibition or eradication of cancer metastasis. For many years, all of our efforts to treat cancer have concentrated on the inhibition or destruction of tumor cells. Strategies both to treat tumor cells (such as chemotherapy and immunotherapy) and to modulate the host microenvironment (including the tumor vasculature) should offer additional approaches for cancer treatment. The recent advances in our understanding of the biological basis of cancer metastasis present unprecedented possibilities for translating basic research to the clinical reality of cancer treatment. (*Endocrine Reviews* 28: 297–321, 2007)

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I. Introduction

ONCE A DIAGNOSIS of primary cancer is established, the urgent question is whether the cancer is localized or whether it has already spread to the regional lymph nodes

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Abbreviations: bFGF, Basic fibroblast growth factor; EGF, epidermal growth factor; EGF-R, EGF receptor; EG-VEGF, endocrine gland-derived VEGF; EPC, endothelial-cell precursor cell; HIF-1 α , hypoxia-inducible factor-1 α ; HSC, hematopoietic stem cell; LPA, lysophosphatidic acid; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; 12(S)-HETE, 12(S)-hydroxyperoxyeicosatetraenoic acid; Stat, signal transducer and activator of transcription; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial cell growth factor; VEGFR, VEGF receptor; VLA-4, very late activation antigen-4.

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and distant organs, where it can produce metastases. Despite improvements in diagnosis, surgical techniques, general patient care, and local and systemic adjuvant therapies, most deaths from cancer result from the progressive growth of metastases that are resistant to conventional therapies. In a large number of patients, metastasis can occur before diagnosis of the primary disease.

Metastases can be located in different organs and in different regions of the same organ. The organ microenvironment can modify the response of metastatic tumor cells to therapy and alter the effectiveness of anticancer agents in destroying the tumor cells without producing undesirable toxic effects. The major obstacle to treating metastasis is the biological heterogeneity of primary neoplasms and metastases. By the time of diagnosis, cancers contain multiple genetically unstable cell populations with diverse karyotypes, growth rates, cell-surface properties, antigenicities, immunogenicities, marker enzymes, sensitivity to various cytotoxic drugs, and abilities to invade and produce metastasis (1–3).

Understanding the mechanisms responsible for the development of biological heterogeneity in primary cancers and metastases and the process by which tumor cells can invade local tissues and spread to distant organs must continue to be a primary goal of cancer research. Only a better understanding will lead to improvements in the design of more effective therapy for cancer metastasis. This review deals with the pathogenesis of cancer metastasis and the contributions of the host vascular system to this process.

II. Pathogenesis of Cancer Metastasis

The process of cancer metastasis is complex and consists of a large series of interrelated steps (shown schematically in

Fig. 1). To produce clinically relevant lesions, metastatic cells must survive all the steps of the process. If the disseminating tumor cell fails to complete even one of these steps, it will not produce a metastasis. The outcome of the metastatic process depends on both the intrinsic properties of the tumor cells and their interactions with host factors (1, 4, 5).

The major steps in the pathogenesis of metastasis are as follows. 1) After the initial transforming event, growth of neoplastic cells must be progressive, with nutrients for the expanding tumor mass initially supplied by simple diffusion. 2) Extensive angiogenesis must occur if a tumor mass is to exceed 1–2 mm in diameter. The synthesis and secretion of proangiogenic factors plays a key role in establishing a neo-capillary network from the surrounding vasculature. 3) Local invasion of the host stroma by some tumor cells occurs by several mechanisms. 4) Thin-walled venules, like lymphatic channels, offer low resistance to penetration by tumor cells and can therefore provide a common pathway for tumor cell entry into the circulation. Although clinical observations have suggested that carcinomas frequently metastasize and grow via the lymphatic system, whereas malignant tumors of mesenchymal origin more often spread by the hematogenous route, the presence of numerous venolymphatic anastomoses invalidates this concept. 5) Small tumor cell aggregates are detached and embolized, but the vast majority of circulating tumor cells are rapidly destroyed. 6) The few tumor cells that can aggregate with host cells and survive the circulation must 7) arrest in the capillary beds of organs, either by adhering to capillary endothelial cells or by adhering to subendothelial basement membrane, which may be exposed. 8) Tumor cells can proliferate within the vessel or

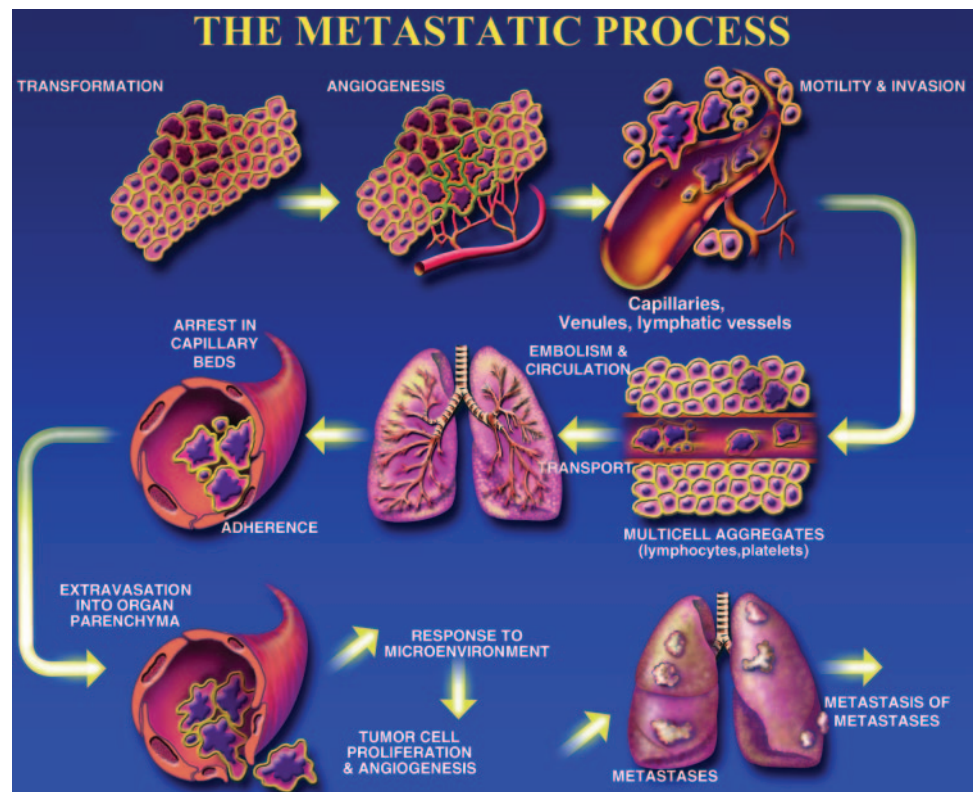
9) extravasate, probably by the same mechanisms that influence initial invasion. 10) Proliferation within the organ parenchyma completes the metastatic process. 11) To continue growing, the micrometastases must develop a vascular network (angiogenesis) and 12) continue to evade the host immune system. The metastatic cells can invade, penetrate blood vessels, and enter the circulation to produce additional metastases, a process known as metastasis of metastases.

The outcome of the metastatic process depends on multiple and complex interactions of metastatic cells with host homeostatic mechanisms (1, 2, 4). Clinical observations of cancer patients and laboratory studies with experimental rodent tumors have shown that certain tumors metastasize to specific organs independent of vascular anatomy, rate of blood flow, and number of tumor cells delivered to each organ. The distribution and fate of hematogenously disseminated, radiolabeled melanoma cells in experimental animals conclusively demonstrated that tumor cells can reach the microvasculature of many organs, but growth in the organ parenchyma occurs in only specific organs (6–12). Similar examinations of the individual steps in metastasis using intravital video microscopy also identified post-extravasation cell growth as the major rate-limiting step in metastasis (13).

A. Role of the organ microenvironment in the pathogenesis of metastasis

As stated above, the outcome of the metastatic process depends on multiple and complex interactions of metastatic cells with host homeostatic mechanisms. In 1889, Stephen Paget (14) researched the mechanisms that regulate organ-

FIG. 1. Pathogenesis of cancer metastasis. The process of cancer metastasis consists of sequential, interlinked, and selective steps. The outcome of each step is influenced by the interaction of metastatic cells with homeostatic factors. Each step of the metastatic process is considered rate limiting in that failure of a tumor cell to complete any step effectively terminates the process. Therefore, the formation of clinically relevant metastases represents the survival and growth of unique subpopulations of cells that preexist in primary tumors.



specific metastasis, or patterns of metastasis by different cancers. To determine whether the organ distribution of metastases produced by different human neoplasms is caused by chance, Paget analyzed 735 autopsy records of women with breast cancer. His research documented a nonrandom pattern of visceral (and bone) metastasis, suggesting that the process is neither random nor due to chance; rather, certain tumor cells (the “seed”) had a specific affinity for the milieu of certain organs (the “soil”). Metastases result only when the seed and soil are compatible (14).

In 1928, Ewing (15) challenged Paget’s “seed and soil” theory proposing instead that dissemination of metastatic cells occurs by purely mechanical factors that result from the anatomical arrangement of the vascular system. However, although hemodynamic and mechanical factors are undeniably important in determining the distribution patterns of several cancers, the mechanical theory does not satisfactorily explain several documented patterns of metastases. For example, choroidal melanoma preferentially metastasizes to the liver and, in doing so, must circumvent several more proximal organs (16). Metastases from clear cell carcinoma of the kidney frequently arise in the thyroid gland, a relationship that cannot be explained by anatomical-mechanical principles. Furthermore, examinations of distribution of metastases in animal models have shown that some tumor cells exhibit specificity for growth in different regions within a single organ (17, 18). Schackert and Fidler (17, 18) noted that injection of K-1735 melanoma cells into the internal carotid artery of mice produced metastases only in the brain parenchyma. However, when these investigators repeated the experiment using B16 melanoma cells, only meningeal growths were observed. In a review of clinical studies on site preferences of metastases produced by different human neoplasms, Sugarbaker (19) concluded that common regional metastatic involvements could be attributed to anatomical or mechanical considerations, such as efferent venous circulation or lymphatic drainage to regional lymph nodes, but that metastasis in distant organs from numerous types of cancers is indeed site-specific.

Data suggesting that tumor cell properties may determine the outcome of metastasis were reported by Zeidman and Buss (20), who demonstrated that tumor cells from different tumors interact differently with the capillary bed of a given organ. Sugarbaker (21) injected tumor cell suspensions from different types of tumors into the same site in rats and observed that each type established its own pattern of metastases. In separate experiments, Fisher and Fisher (22) demonstrated that tumor cells can traverse different organs at different rates. Strong experimental evidence that tumor cells home to and grow in particular organs was first reported for Cloudman melanoma by Kinsey (23) and then for murine sarcoma by Sugarbaker *et al.* (24). In both studies, neonatal tissue was implanted in the thighs of syngeneic mice. After the intraarterial or sc injection of lung-colonizing tumor cells, metastatic foci developed in the *in situ* lung as well as the grafted lung, but not in other grafted organ controls.

The preferential growth of B16 melanoma metastases in specific organs was studied by Hart and Fidler (25). After the iv injection of B16 melanoma cells into syngeneic C56BL/6 mice, tumor growths developed in the *in situ* lungs and in

grafts of pulmonary or ovarian tissue implanted in either skin or muscle. In contrast, neoplastic lesions failed to develop in control grafts of similarly implanted renal tissue or at the site of a surgical trauma. Parabiosis experiments suggested that the growth of the B16 melanoma in ectopic lung or ovary tissue resulted from the immediate arrest of circulating neoplastic cells and not from shedding of malignant cells from foci growing in the *in situ* lungs. Quantitative analysis of tumor cell arrest and distribution using cells labeled with [¹²⁵I]-5-iodo-2'-deoxyuridine indicated that the growth of tumors in the implanted organs was not due to an enhanced initial arrest of B16 cells. No significant differences in immediate tumor cell arrest were detected between implanted fragments of lungs (tumor positive) and kidney (tumor negative) or between organ-bearing and contralateral control limbs.

The introduction of peritoneovenous shunts for palliation of malignant ascites has provided a similar opportunity to study some of the factors affecting the spread of malignant cells in humans. Tarin *et al.* (26) have described the outcome in patients with malignant ascites draining into the venous circulation, with the resulting entry of viable tumor cells into the jugular veins. Good palliation with minimal complications was reported for 29 patients with different neoplasms. The autopsy findings in 15 patients substantiated the clinical observations that the shunts do not significantly increase the risk of metastasis. In fact, despite continuous entry of millions of tumor cells into the circulation, metastases in the lung (the first capillary bed encountered) were rare.

Reports suggest that unique factors produced by each of the individual tissues in the body may exert differential effects on tumor cell growth. For example, Nicolson and Dulski (27) reported that the growth rate of ovary-colonizing B16 melanoma sublines was stimulated by ovary-derived factors significantly more than by factors produced in other tissues. In other instances, the tissue microenvironment may act as a negative regulator of tumor cell growth and much recent consideration has been directed toward defining the molecular basis of tumor dormancy, *i.e.*, that variable period of time during which tumor cells may exist in an inactive state. That tumors frequently recur many years after treatment is a well-recognized characteristic of malignant disease. Cell cycle arrest (28) and immune surveillance (29) are two factors that have been implicated in tumor dormancy. Studies utilizing fluorescently labeled tumor cells implanted in different organs of the mouse have shown that tumor cells are capable of existing as a single entity for a prolonged period of time in some organs (30). However, when a dormant cell is removed from the inhibitory influences of the organ microenvironment, it quickly regains metastatic competency (31).

The inability of some tumor cells to initiate an effective angiogenic response is also suspected of contributing to tumor dormancy (32). Several studies have determined that expression of proangiogenic cytokines by malignant cells is under the regulation of the tissue microenvironment. For example, Takahashi *et al.* (33) examined the growth of gastric cancer cells that were implanted into either the stomach or subcutis of nude mice. Tumors growing in the stomach expressed significantly more vascular endothelial cell growth

factor (VEGF)/vascular permeability factor and had a greater vascular density than the ectopically placed tumor, and moreover, only those tumors implanted into the stomach were metastatic. A study comparing the growth of human colon cancer cells that were implanted into either the cecum or sc space yielded similar results (34). Microenvironmental factors have also been shown to influence expression levels of basic fibroblast growth factor (bFGF), a growth factor that controls the angiogenic switch of some tumors (35). When human renal cell carcinomas were implanted into different organs in nude mice, the expression of bFGF was found to be 20-fold higher in tumors implanted in the kidney than those implanted in sc tissues (36). The tumors that were implanted into the skin contained very few blood vessels and were characterized by a stroma that was rich in the angiostatic protein interferon- β , whereas no interferon- β was expressed in renal cell carcinomas implanted in the kidney.

B. Development of a genetically unstable primary tumor

Cancer is a genetic disorder that is characterized by excessive proliferation. The genetic clonality model proposes that cancer evolves from a single cell that has undergone multiple rounds of cell division, mutation, and selection (37). The genetic information contained in cellular genes may be altered by errors that arise during cell division or by physical carcinogens (such as UV or ionizing radiation) or chemical carcinogens (such as 2-naphthylamine or N-nitrosamine) (38). Genetic modifications that render cells unresponsive to the normal signaling cues that regulate cell division lead to the generation of preneoplastic lesions, and additional mutations can give rise to clonal variants that proliferate into cancer (39). Estimates predict that during the multistep process of tumorigenesis, a cell must acquire at least six mutations to become malignant (40). Malignant cells are characterized by a phenotype that includes self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, extensive replicative potential, ability to avoid programmed cell death (apoptosis), sustained angiogenesis, and ability to invade and produce metastases (41).

Mutations associated with the development of tumors occur in two classes of genes—protooncogenes and tumor-suppressor genes—both of which play critical regulatory roles in cell proliferation. Point mutations, gene amplification, and chromosomal rearrangements alter structural or functional properties of protooncogenes and lead to activation of oncogenes. A classification scheme places oncogenes into five groups based on functional and biochemical properties of their normal counterparts (protooncogenes): growth factors, growth factor receptors, signal transducers, transcription factors, and others, including programmed cell death regulators (42). Whereas the products of oncogene activation signal for enhanced cell proliferation, tumor suppressor genes encode proteins, such as p53 or retinoblastoma protein, that impede cell growth. Hence, mutations that target tumor suppressor genes result in their inactivation. Studies indicate that expression of the malignant phenotype requires a combination of oncogene activation and tumor suppressor inactivation (43).

A number of recent studies have investigated the identity of the cells responsible for the genesis of cancer. Emerging

evidence suggests that stem cells or their immediate progeny may give rise to various types of tumors as a result of a dysregulated self-renewal process (44). Proponents of the “stem cell hypothesis” of cancer maintain that certain biological properties of stem cells, such as inherent longevity and the ability to self-replicate, make stem cells the ideal candidate to accumulate the full complement of mutations required for tumorigenesis (44–46). Self-replication of normal stem cells is an asynchronous process in which a stem cell gives rise to an exact duplicate of itself and a committed progenitor cell that will proliferate into differentiated progeny (47). Studies on stem cells have determined that the self-replication process is tightly regulated by signals transmitted from the stem cell niche, a specialized microenvironment composed of groups of cells that function in stem cell maintenance (48, 49). Self-renewal and differentiation programs in normal stem cells are regulated by signaling through various components of the Wnt (50), Hedgehog (51, 52), and Notch pathways (53, 54). Oncogenic mutations that eliminate stem cell dependence on the niche for proliferation and differentiation information may promote expansion of the pool of self-renewing cells in which further mutations can accumulate and give rise to cancer stem cells (46). The molecular mechanism responsible for the uncoupling of stem cells from niche signaling remains unknown.

Evidence that stem cells may be responsible for the genesis of some tumors comes from investigations of hematopoietic malignancies. Bonnet and Dick (55) demonstrated that human leukemic cells expressing the primitive progenitor CD34⁺CD38⁻ phenotype could be transferred to nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice to produce leukemia. The fraction of cells with leukemia-initiating activity was small (0.1–1.0% of all cells), and cells deficient in CD34⁺CD38⁻ were unable to form tumors. Recent data suggest that a small population of cells with “stem-like” characteristics may be responsible for tumors originating from the breast and central nervous system. Al-Hajj *et al.* (56) isolated CD44⁺CD24^{-/low}Lin⁻ tumor cells from eight of nine patients with breast tumors and determined that this subpopulation of cells was responsible for tumorigenesis. When the tumorigenic cells were injected into mice, the cells gave rise to additional CD44⁺CD24^{-/low}Lin⁻ tumorigenic cells as well as phenotypically diverse nontumorigenic cells. The tumor heterogeneity observed in these mice recapitulated the complexity of the primary tumors from which the tumorigenic cells had been derived. Accumulating evidence suggests that multiple regions in the adult brain, including the subventricular zone, lining of the lateral ventricles, dentate gyrus, hippocampus, and subcortical white matter, contain populations of neural stem cells and glial progenitor cells (57). Recently, Singh *et al.* (58) isolated CD133⁺ tumor cells from human gliomas and transplanted the cells into NOD/SCID mice to demonstrate that these cells were the tumor-initiating cells. The transplanted CD133⁺ cells gave rise to a heterogeneous tumor in which the majority of tumor cells were CD133⁻. Purified populations of these CD133⁻ cells did not form tumors when implanted into the mouse brain.

It has been over a century since Cohnheim (59, 60) pro-

posed the “embryonic theory” of cancer that postulated that human tumors arise from embryonic cells that persevere in tissues without reaching maturity. Over recent years, there has been a growing appreciation that a relatively small population of cells that possess self-renewal properties and cell surface markers characteristic of stem cells may be responsible for the progression of some tumors. The recognition that the signaling pathways that regulate normal stem cell division (*i.e.*, Notch, Wnt, Hedgehog, Bmi1) are dysregulated in a number of human tumors also lends some support to the cancer stem cell hypothesis. Nevertheless, despite its appeal, the notion that malignant growth is a product of a dysfunctional stem cell is at the center of considerable debate, and a number of issues remain unresolved. For example, it still remains unclear whether oncogenic mutations arise within the stem cell population, transit-amplifying progenitor cells, or whether a transforming event induces a stem cell-related phenotype in a more committed cell. In addition, there is a paucity of data in the literature concerning the role of stem cells in metastasis. If the hypothesis is correct that the only cell type within a primary mass that possesses tumorigenic potential is the cancer stem cell, then any clinically relevant metastases must originate from a cancer stem cell. One may argue that, for most types of tumors, understanding the molecular make-up of the metastasizing cell population is more urgent than continued investigations of primary lesions. To provide some insight into the genetic background of metastases, Glinsky *et al.* (61) examined patterns of gene expression in both murine models and human tumors and reported that metastases express an 11-gene molecular signature that is strikingly similar to a stem cell expression profile. Moreover, it was determined that the set of 11 transcripts could be used to predict clinical outcome in a broad range of human tumors.

III. Vascular System in Metastasis

A. Angiogenesis

Irrespective of the origin of the tumor cell, once it becomes refractory to the regulatory mechanisms that control normal cell division and differentiation, the primary determinant that governs its progression and survival is its proximity to a vascular supply. Indeed, data derived from examinations of human lung cancer brain metastases indicate that tumor cell division takes place within 75 μm of the nearest blood vessel, whereas tumor cells residing beyond 150 μm from a vessel undergo programmed cell death (7) (Fig. 2). These measurements are in agreement with the diffusion coefficient of oxygen in tumor tissue, which is approximately 120 μm (62). Hence, simple diffusion of oxygen may support the viability of tumor cells within a mass smaller than 1 mm in diameter. Any additional expansion must be preceded by an increase in vascular density. Oncogene activation and loss of cell cycle regulation signal for persistent tumor cell division and, ultimately, the metabolic demands of the expanding mass will exceed blood flow delivery. An extensive body of evidence generated over a period of several decades has concluded that the primary compensatory mechanism employed by tumor cells to offset increasing metabolic pressures involves the recruitment of resident microvascular endothelial cells to form new vascular networks, a process known as angiogenesis (63, 64).

Angiogenesis refers to the development of new blood vessels from the preexisting vasculature. The generation of a vascular supply is essential for embryonic development, maintenance of reproductive function, and wound repair (65–67). In addition, angiogenesis plays a key role in the initiation and perpetuation of a number of pathophysiological processes, including arthritis, diabetic retinopathy, macular degeneration, and neoplasia (68–70). Unlike the vascularization that accompanies highly regulated physiological

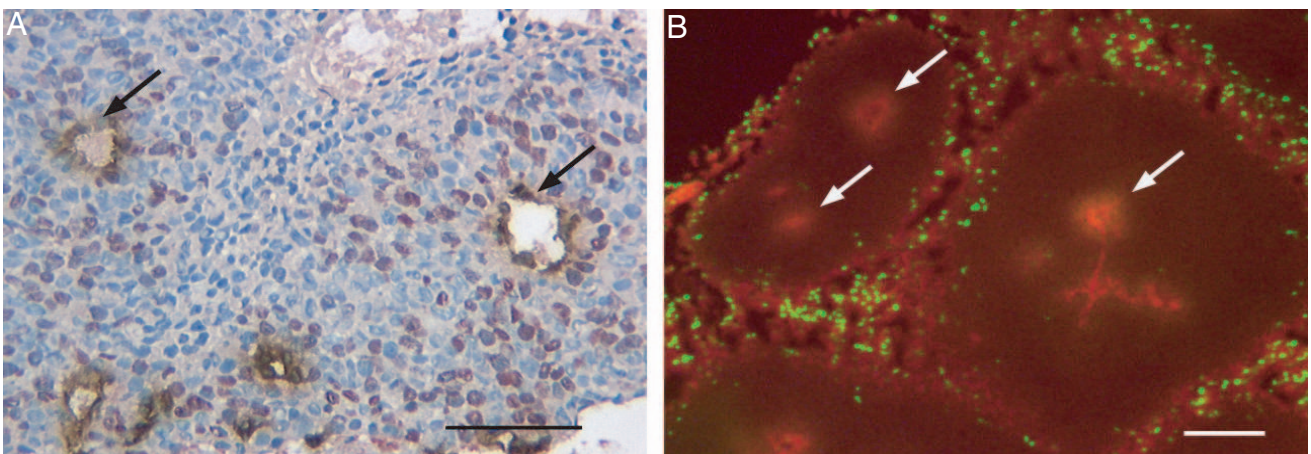


FIG. 2. Location of dividing and apoptotic tumor cells in relation to blood vessels in brain metastases. A, Autochthonous human lung-cancer brain metastases. Dividing cells were labeled with antibody directed against 5-bromo-2-deoxyuridine and are stained in red. The arrows point to blood vessels. B, Autochthonous human lung-cancer brain metastases. To determine the distance of apoptotic cells from the nearest blood vessel, tumor vessels were labeled for CD31 antigen and are stained in red, and apoptotic cells (terminal dUTP nick-end labeling-positive) are stained bright green. The distribution of dividing and apoptotic cells was studied with the Euclidean distance map (EDM); dividing cells were always within 100 μm of the nearest vessel, and apoptotic cells were located 160–170 μm from the nearest vessel. Scale bars, 100 μm . [Reproduced from I. J. Fidler *et al.*: *Lancet Oncol* 3:53–57, 2002 (7) with permission from Elsevier.]

processes, angiogenesis associated with tumor growth is incessant. This observation has led Dvorak (71) to characterize tumors as “wounds that do not heal”.

The induction of angiogenesis is a consequence of an imbalance between multiple inhibitor and stimulator molecules and is referred to as the “angiogenic switch” (35, 72). Normal tissues are exposed to an excess of inhibitor molecules that maintain the vascular endothelium in a quiescent, nonproliferating state. Measurements of cell proliferation in non-diseased tissues indicate that the turnover time of endothelial cells may be measured in years (73). Activation of the angiogenic switch may occur at any stage of tumor development; however, inception is usually synchronized with increasing metabolic pressures, oncogene activation, or mutation of tumor suppressor genes (72). For example, loss of the wild-type allele of the p53 tumor suppressor gene results in reduced production of the angiostatic factor thrombospondin-1 (74), and activation of the ras oncogene (75) or inactivation of the von Hippel-Lindau tumor suppressor gene (76, 77) increases expression of VEGF, a potent proangiogenic cytokine. Investigations conducted over the past several decades have identified a number of other angiogenic molecules, including members of the fibroblast growth factor family, IL-8, epidermal growth factor (EGF), angiogenin, and others (67, 73, 78). These proteins activate angiogenic programs in endothelial cells that signal a number of biological responses, including directional migration, invasion, cell division, proteolysis, expression of antiapoptotic proteins, and ultimately, new capillary formation (64, 66, 69, 70, 79, 80).

Studies have shown that the intensity of the angiogenic response varies considerably between different types of tumors. For example, measurements of endothelial cell division in human cancers indicate that angiogenesis accompanying the progression of glioblastoma and renal cell carcinoma is significantly greater than the blood vessel development that occurs during the growth of lung or prostate tumors (81). In general, slow-growing benign tumors usually contain few vascular structures, whereas fast-growing malignant neoplasms are highly vascular (2). A greater vascular density increases the probability that tumor cells can gain access to the systemic circulation to reach distant organs (82). Several studies have reported that an increased microvascular density in the “hot spots” of most intensive neovascularization is a valuable prognostic indicator for tumors arising from the breast (83), prostate (84), bladder (85), stomach (86), and colon (87).

Despite the intensity of the angiogenic response, the rate of cell division in neoplasms is several orders of magnitude greater than the rate of neovascularization and hence, insufficient blood flow is a common feature of many tumors. Studies conducted in experimental animal tumors suggest that the reduction in blood flow can be profound compared with measurements in normal tissues. This was emphasized by experiments conducted by Gullino and Grantham (88), who demonstrated that tumor blood flow to animals bearing ovarian tumors was 50 times less than the blood flow to normal tissues. As the tumor expands, the vascular space becomes a progressively smaller component of the total mass, so the microenvironment of tumors is often hypoxic. In fact, it is estimated that 50–60% of locally advanced solid

tumors have hypoxic or anoxic regions that are heterogeneously distributed within the tumor (89, 90). The decline in oxygen tension observed in some tumors, such as head and neck carcinomas, has been shown to function as a selective pressure that leads to the proliferation of cells with enhanced metastatic potential (91).

Restoration of oxygen homeostasis can occur by activating the transcription factor hypoxia-inducible factor-1 α (HIF-1 α) to initiate the transcription of genes encoding angiogenic growth factors (92, 93). Blouw *et al.* (94) recently demonstrated that the anatomic location of the tumor determines whether continued proliferation of the neoplasm will depend on HIF-1 α -mediated angiogenesis. Specifically, the investigators noted that tumors residing in tissues that contain an inherently high microvascular surface area (such as brain) may be less dependent on the neovascularization response than tumors located in anatomic regions with fewer vascular structures (such as sc space). Nevertheless, HIF-1 α has been localized to hypoxic regions of tumors, and overexpression has been reported in several primary human cancers and their metastases (95–97).

Perhaps the most intensely studied gene targeted by HIF-1 α activation is VEGF. VEGF belongs to a gene family that includes placental growth factor, VEGFB, VEGFC, and VEGFD (98). These proteins transduce their effects by binding to three distinct VEGF receptors: VEGFR1 (Flt-1), VEGFR2 (KDR/Flk-1), and VEGFR3 (Flt-4) (99, 100). VEGFA isoforms are considered the prototypic angiogenic molecules by virtue of their ability to induce most of the processes required for the assembly of new blood vessels (migration, protease production, and proliferation) (99). In addition, VEGF increases the permeability of blood vessels by stimulating the functional activity of vesicular-vacuolar organelles, clusters of cytoplasmic vesicles and vacuoles located in microvascular endothelial cells (100). The hyperpermeability of tumor microvessels induced by VEGF expression is thought to facilitate tumor progression by generating an extravascular fibrin gel that acts as a substrate for endothelial and tumor cell growth (101). VEGF also mediates endothelial cell survival by up-regulating the phosphatidylinositol-3 kinase/Akt signal transduction pathway (102) and stimulating expression of the antiapoptotic proteins Bcl-2 and A1 (79).

Other target genes activated in response to HIF-1 α signaling are those that encode the polypeptide chains of platelet-derived growth factor (PDGF) (103). PDGF is a family of cationic homo- and heterodimers of disulfide-bonded A- and B- chains (104), which are synthesized as precursor molecules that assemble into dimers and undergo proteolytic processing (105). To date, five PDGF isoforms have been identified: PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD. These isoforms mediate their effects by binding to two tyrosine kinase receptors, PDGF-R α and PDGF-R β . Studies evaluating the role of PDGF in the neovascularization of tumors suggest that the functional activity of PDGF is, to a large extent, determined by the anatomical location of the tumor in question. Results generated from various experimental tumor models emphasize the diverse roles that tyrosine kinase receptors may assume in different vascular beds. For example, in tumors growing in the pancreas, PDGF has been shown to stabilize developing vascular networks by

recruiting pericytes to support the immature blood vessel wall (106). In tumors originating in the central nervous system, PDGF promotes angiogenesis, in part, by stimulating the release of VEGF from the tumor-associated endothelium (107). In contrast, tumors in the skin rely on PDGF signaling to regulate the level of interstitial fluid pressure in the tumor (108). In prostate cancer bone metastasis, PDGF functions as a survival factor for tumor endothelial cells by activating the intracellular effectors MAPK and Akt (109, 110). Small-molecule inhibitors such as imatinib (STI571 or Glivec; Novartis Oncology, Basel, Switzerland) that selectively inhibit activation of PDGF-R signal transduction have been shown to have a dramatic effect in the treatment of chronic myelogenous leukemia (111, 112). Consequently, much effort is directed toward determining whether PDGF-R signaling is essential for the progression of solid neoplasms.

B. Organ-specific endothelium

Given that the vascular endothelium is widely regarded as a structurally and functionally heterogeneous tissue (113–116), the fact that PDGF ligands elicit tissue-specific responses from endothelial cells is not surprising. Indeed, endothelial cells from different regional circulations exhibit diversity with respect to antigenic composition, production of vasoactive factors, metabolic properties, response to growth factors, and susceptibility to pathological insult (117–119). Studies examining the molecular basis of heterogeneity in the vascular system have concluded that endothelial cell diversity is the product of both genetic (120) and environmental (121, 122) influences.

Several studies have sought to identify differentially expressed endothelial cell determinants that may be exploited to selectively deliver therapeutic agents to a given tissue. Efforts attempting to concentrate drugs in a distinct anatomic region are important in that their success could potentially eliminate much of the global toxicity associated with standard drug-delivery approaches. Examinations of the vascular endothelial cell surface using phage-display peptide libraries have shown that the blood vessels that supply both normal (123, 124) and tumor (125) tissues express unique endothelial cell receptors and that these surface specializations can support site-directed delivery of drug to tumors (125). McIntosh *et al.* (126) discovered biochemical and structural differences in the composition of transport vesicles of different endothelia and found that this discriminating feature could be used to transport immunotoxin exclusively to the lung. Hood *et al.* (127) devised an elegant strategy to therapeutically target tumor-associated endothelial cells. Their tumor vascular targeting strategy was directed at $\alpha_v\beta_3$ integrin, a cell surface receptor that is up-regulated on dividing endothelial cells (128, 129). By coupling an $\alpha_v\beta_3$ -targeting ligand to cationic nanospheres, the investigators were able to localize nanoparticles to the blood vessels perfusing tumors implanted in mice. Moreover, when the investigators conjugated cDNA encoding mutant Raf-1 to the tumor-targeting nanospheres, the growth of primary tumors and experimental metastases was significantly repressed.

Separate investigations have been designed to identify factors that are responsible for stimulating endothelial cell

proliferation in different anatomic regions. Results from these studies suggest that the efficacy of VEGF in promoting angiogenesis varies considerably among different organs (130) and that some organs synthesize unique endothelial cell mitogens that possess very restricted activity (131). LeCouter *et al.* (131) identified endocrine gland-derived VEGF (EG-VEGF) while screening a library of human proteins for their ability to induce proliferation of capillary endothelial cells derived from the adrenal cortex. The 8.6-kDa EG-VEGF protein displayed properties similar to those of VEGF, but the activity of EG-VEGF was restricted to capillary endothelial cells derived from endocrine glands. To study how endothelial cells from different tissues contribute to angiogenesis and metastasis, we generated a broad panel of microvascular endothelial cells from various organs of *H-2K^b-tsA58* transgenic mice (132). Cells derived from these mutant mice all harbor a temperature-sensitive SV40 large T antigen that allows the user to regulate the level of cell differentiation (133). cDNA expression profiles generated on the endothelial cells predicted significant organ-specific differences in expression levels of tyrosine kinase receptors, chemokine receptors, and proteins that regulate the efflux of toxic substrates; these were confirmed at the protein level (134). For example, we noted that endothelial cells derived from the mouse brain expressed measurable levels of PDGF-R β , the chemokine receptor CXCR-2, and P-glycoprotein, whereas endothelial cells from the pulmonary circulation did not express detectable levels of these proteins. The organ-derived endothelial cells also exhibited vast differences in response to stimulation with endothelial cell mitogens. Endothelial cells originating from the brain and liver showed the greatest increase in cell division in response to bFGF, whereas EGF was the most potent mitogen for endothelial cells derived from the lung and uterus (134). Cerebral endothelial cells were found to possess the greatest number of redundant growth factor signaling pathways. The number of growth factor signaling pathways present in these cells may be a reflection of the deleterious consequences that ensue upon cessation of cerebral blood flow. In any event, this observation emphasizes the difficulties in attempting to inhibit angiogenic responses in the brain.

C. Tumor-specific endothelium

Molecular profiles of tumor-associated endothelial cells constructed by serial analysis of gene expression indicated significant genetic differences between tumor-associated endothelial cells and endothelial cells in adjacent normal blood vessels (135). One distinguishing feature of tumor endothelial cells is their tendency to express the EGF receptor (EGF-R) (136). Indeed, results from our laboratory have demonstrated that EGF-R is phosphorylated on endothelial cells in tumor blood vessels in both xenograft models and humans when adjacent tumor cells express the EGF-R ligands TGF- α and/or EGF (137, 138). The activation of EGF-R on endothelial cells of tumor blood vessels appears to play an essential role in tumor progression inasmuch as pharmacological suppression of this signaling cascade in experimental tumors inhibits the growth of primary lesions and, more importantly, reduces the frequency of metastasis (137–140).

To pattern the phenotype of tumor endothelial cells and examine the effects of perpetual stimulation of EGF-R on endothelial cells, we created a constitutively active chimeric EGF-R by fusing the entire intracellular domain of the EGF-R to the N-terminus of the CD3 ζ component of the T cell receptor signaling complex (140). The chimeric receptor, CD3-EGF-R, was then stably introduced into brain endothelial cells, where it signaled for enhanced migration, synthesis of matrix metalloproteinase-9 (MMP-9), invasion, and aggressive growth. An examination of intracellular signaling pathways in brain endothelial cells expressing CD3-EGF-R indicated that signal transducer and activator of transcription 3 (Stat3) was responsible for the induction of angiogenic programs in these cells.

The Stat proteins belong to a family of transcription factors that are activated when certain ligands, including growth factors, cytokines, and hormones, bind to their respective receptors. Stat activation results in the generation of homo- and heterodimers that are translocated to the nucleus, where they bind to target gene promoters (141). Growing evidence suggests that constitutively activated Stat3 expression is a common feature in a variety of tumors including those arising from the head and neck, brain, breast, lung, and other tissues (142–145). Several tumors appear to be critically dependent on Stat3 signaling for growth and survival; not surprisingly, Stat3 is attracting much attention as a potential target for therapeutic intervention (146). Our finding that Stat3 is an important regulator of angiogenesis in some endothelial cells suggests that inhibition of Stat3 signaling in tumors that produce EGF-R ligands may control tumor growth by directly affecting tumor cell proliferation and by limiting the angiogenic response of the tumor-associated blood vessels.

Although most investigations examining the neovascularization of tumors have concentrated on angiogenesis, more recent evidence suggests that hematopoietic stem cells (HSCs) and endothelial-cell precursor cells (EPCs) may also play an important role in the generation of new vascular networks. Asahara *et al.* (147) were the first to isolate EPCs from human peripheral blood and demonstrate that these cells could traffic to areas of ischemic tissue and contribute to developing vascular structures. EPCs can be distinguished from mature circulating endothelial cells by virtue of their unique expression of cell-surface markers including VEGFR2, AC133, CXCR4, and CD14 (148). Studies have shown that recruitment of HSCs and EPCs from the bone marrow to tumors is initiated in response to tumor-secreted products that stimulate activation and secretion of MMP-9 by hematopoietic cells in the bone marrow (149). MMP-9 activation leads to the liberation of soluble KIT ligand, a factor that is responsible for coordinating the final two steps in the recruitment process. Soluble KIT first stimulates division of progenitor cells and then provides the cells with the directional information necessary to guide their entry into the peripheral circulation.

Direct evidence of a role for HSCs and EPCs in the vascularization of tumors comes from experiments conducted in mutant mice that are deficient in Id proteins. Mice with the Id1^{+/-}Id3^{-/-} phenotype lack the ability to mount an angiogenic response and are therefore unable to support tumor

growth (150). However, when these mice are transfused with bone marrow stem cells from wild-type donor mice, angiogenesis and tumor growth are restored (151). Cooperation from both VEGFR1 and VEGFR2 appears to be essential in the neovascularization response because neutralizing antibodies that block both of these receptors in Id1^{+/-}Id3^{-/-} mutants is required to cause full-scale vascular disruption and tumor cell death. Whether HSCs and EPCs contribute to tumor vessels appears to depend on the tumor type in question. Most studies conclude that these cells represent only a small fraction (6–10%) of the tumor-associated blood vessels and that vessels containing progenitor cells are formed during the early stages of tumor growth (152). Despite the relatively low number of progenitor cells that contribute to tumor blood vessels, evidence suggests that the homing properties of these cells may be exploited for therapeutic purposes. Indeed, EPCs that were stably transfected with thymidine kinase (153) or the soluble truncated form of VEGFR-2 (154) were found to successfully traffic to the tumor neovasculature and significantly impair tumor growth.

Although induction of angiogenesis appears to be a rate-limiting step in the growth and spread of most neoplasms, results from recent studies suggest that there are certain subsets of tumors that are capable of continued growth in the absence of angiogenesis (155). Instead of stimulating the development of new vascular networks to support their growth, these tumors meet their metabolic requirements by residing in the vicinity of preexisting blood vessels. Reports have documented angiogenesis-independent growth in murine models of melanoma brain metastasis (156) and glioma (157) and in human non-small-cell lung tumors (158). Intuitively, this pattern of tumor growth should be impervious to therapeutic interventions that are designed to target dividing endothelial cell populations. It remains unclear whether human tumors revert to angiogenesis-independent growth patterns when confronted with antiangiogenic agents. An additional dilemma regarding cancer therapies that target only angiogenesis is the recent finding that therapy designed to inhibit signaling initiated by a single endothelial cell mitogen results in up-regulation of redundant tumor factors that are capable of sustaining endothelial cell growth and survival (159).

To gain access to the lymphatic or blood vascular systems and establish distal metastases, tumor cells must penetrate the stroma that includes the basement membrane. Indeed, a primary histopathological feature of malignant tumors is the disruption of the epithelial basement membrane and the presence of cancer cells in the stromal compartment (160). In contrast, benign lesions are always characterized by a continuous basement membrane that separates the neoplastic epithelium from the stroma (160, 161). The process of tumor invasion is initiated by a loss of cell-to-cell cohesive forces. In epithelial cells, homotypic cell adhesive interactions are maintained by epithelial cadherin (E-cadherin), a transmembrane glycoprotein that is anchored to the actin cytoskeleton by cytoplasmic proteins called catenins (162). E-cadherin is localized at the epithelial junctional complex, where it maintains the organization and morphogenesis of epithelial tissues (163). Down-regulation of E-cadherin is associated with a decrease in cellular and tissue differentiation and an in-

crease in carcinoma grade (164–168). Similarly, when invasive cells are transfected with E-cadherin cDNA, their ability to invade is abolished (169). E-cadherin can be functionally inactivated during tumor progression by somatic mutation or through down-regulation of gene expression by promoter methylation and/or transcriptional repression (170).

Liotta (171) has advanced a three-step hypothesis to explain the cellular and molecular mechanisms that allow tumor cells to traverse basement membrane barriers and invade surrounding tissues. This hypothesis suggests that tumor cells use repetitive cycles of attachment, local proteolysis, and migration during the invasion process. Although the basement membrane is permeable to most molecules, it is impervious to cells. Invasive tumor cells mediate their attachment to the basement membrane using laminin and integrin receptors, which are frequently overexpressed on tumor cells with high metastatic potential (172–174). Localized proteolysis is initiated at the tumor cell-basement membrane interface in a process that signifies the transition from a benign carcinoma *in situ* to a malignant invasive tumor cell (175). Efforts to study the proteolytic interactions that occur between tumor cells and basement membrane components have been facilitated by the development of synthetic membrane barriers that permit detailed analyses in a controlled environment. Data generated from these systems indicate that tumors that manufacture elevated levels of type IV collagenase (gelatinase, MMP) possess a greater metastatic potential than their benign counterparts. Several aggressive human tumors, including carcinomas, melanomas, hepatomas, fibrosarcomas, and reticulum cell sarcomas, were found to produce higher levels of type IV collagenase than do benign cells (176). Persuasive evidence that type IV collagenase is critical to the invasion process has been obtained from experiments in which inhibitors of the enzyme routinely abolish the invasive phenotype (177).

D. Hematogenous metastasis

To produce metastases via the systemic circulation, tumor cells must survive transport in the circulation, adhere to the microvascular wall of distal tissues, and either grow locally or invade the vessel wall and grow in the organ parenchyma. Studies have shown that most tumor cells that enter the bloodstream are eliminated rapidly, so the mere presence of tumor cells in the circulation does not predict that metastasis will occur (6, 8, 178). Fidler (6) used radiolabeled tumor cells to demonstrate that after 24 h in the circulation, less than 0.1% of the tumor cells were viable. Moreover, less than 0.01% of tumor cells placed in the circulation eventually survived to generate lung metastases. These results emphasize that metastasis is a highly inefficient pathological process.

Studies investigating the relationship between angiogenesis and metastasis of some tumors have determined that tumor cell entry into the vascular system is closely associated with the neovascularization process. Liotta *et al.* (82) measured the number of tumor cells released from perfused murine tumors and concluded that the dynamics of hematogenously initiated metastasis depend strongly on the entry rate of tumor cell clumps into the circulation, which is in turn dependent upon the extent of angiogenesis. Capillaries lack

the muscular elements associated with larger-caliber vessels, and those formed during tumor progression appear to be a primary entry point for intravasating cells. Angiogenic vessels are inherently leaky (99, 102) and possess a fenestrated endothelium (179, 180), factors that may contribute to their ability to support penetration by tumor cells.

Accumulating evidence suggests that tissue-specific gradients of chemoattractant cytokines, referred to as chemokines, play an important role in determining the patterns of metastasis observed in some tumors. Chemokines are small (8–10 kDa) proteins that are classified into one of four families (CXC, CC, C, and CX3C), depending on the configuration of the cysteine residues located at the amino terminus (181). Chemokines are expressed by a number of different cell types, including fibroblasts, macrophages, leukocytes, vascular endothelial cells, and epithelial cells, and expression levels are generally enhanced during inflammatory responses. Chemokine receptors belong to the G protein-coupled receptor family and are differentially expressed by the various leukocyte subsets (182). Hence, chemokine receptor-ligand interactions determine the composition of the inflammatory infiltrate that is characteristic of different pathologies, including cancer (183).

Much of the data implicating chemokines in tissue-specific metastasis are derived from examinations of the interactions between the CXCR4 chemokine receptor and its ligand, stromal cell-derived factor-1 (SDF-1: CXCL12) (184). Initial reports were focused on the role of CXCR4 in breast cancer after it was determined that CXCL12 was constitutively expressed by stromal fibroblasts in target organs of metastasis (*i.e.*, bone, liver, lung, and lymph node), but not in other tissues (185). That CXCL12 provides the directional information required for tumor cell homing to target organs of metastasis is supported by results from experimental models of breast cancer in which neutralization of CXCR4 signaling abrogates lung and lymph node metastasis (185–187). Activation of CXCR4 on breast cancer cells has been shown to stimulate a number of cellular responses that are critical for metastasis formation, including actin polymerization, pseudopodia formation, chemotaxis, synthesis of proteolytic enzymes, and invasion (188). In addition, stimulation of CXCR4 on tumor cells promotes activation of integrin receptors (189), thereby increasing the affinity of cells for the microvascular endothelial surface (190).

At present, the chemokine classification scheme consists of more than 40 chemokines and 18 different receptors. Whereas the interactions between CXCR4 and CXCL12 play an important role in localizing tumor cells to the more common sites of metastasis, other receptor-ligand pairs appear to coordinate tumor cell recruitment to different anatomic regions. In fact, studies have shown that one can actually determine the site of metastatic relapse in breast cancer patients by examining the distribution of chemokine receptors in the primary mass (191). For example, primary tumors that predominantly express CX3CR1 preferentially spread to the brain, whereas tumors that express CCR6 are more likely to metastasize to the pleura (191). Expression of CCR7 by breast (185) or melanoma (185, 192) cells has also been shown to be an important determinant in mediating skin metastasis.

Once tumor cells reach their target organ of metastasis,

they facilitate their retention in that tissue by forming stable adhesive interactions with the microvascular endothelial cell surface. The arrest of tumor cells in distant microvascular blood vessels is regarded as a key, rate-limiting step in metastasis and may occur by passive (*i.e.*, steric hindrance) or active (*e.g.*, selective adhesive interactions) mechanisms. The ability of blood-borne malignant tumor cells to adhere to specific endothelium and to produce endothelial cell retraction also plays an important role in mediating the site-specific distribution of metastasis (193, 194). Several studies have shown that many tumor cells mediate their adhesion to the vascular endothelium by using mechanisms similar to those used by leukocytes. E-selectin is a cytokine-inducible endothelial cell glycoprotein that is responsible for directing the initial localization of neutrophils to inflammatory tissues (195). Studies examining the contribution of E-selectin to malignant disease have shown that the entry of colorectal carcinoma cells into the hepatic circulation stimulates cytokine production from Kupffer cells, which leads to *de novo* synthesis of E-selectin by sinusoidal endothelial cells (196). Colorectal carcinoma cells then use their tetrasaccharide ligands, sialyl Lewis x and sialyl Lewis a, to form adhesive bonds with E-selectin to be retained in the liver. Expression of the sialyl Lewis a and x antigens on colorectal carcinoma cells is positively correlated with their metastatic potential (197), and blockade of E-selectin in the liver microcirculation has been shown to significantly reduce the frequency of liver metastases in experimental animal models (198). There is also evidence to suggest that prostate tumor cells exploit E-selectin to promote their trafficking to the bone (199, 200). The bone microcirculation differs from that of other vascular beds in that E-selectin is constitutively expressed on endothelial cells, where it directs the recirculation of hematopoietic progenitor cells to the bone (201). The constitutive expression of E-selectin observed on bone microvascular endothelial cells suggests that prostate tumor cell adhesion in this tissue is independent of cytokine production.

Accumulating evidence suggests that some nonepithelial tumors, such as melanomas, may use their integrin receptors to form adhesive bonds with the microvascular endothelium. Vascular cell adhesion molecule-1 (VCAM-1) is an endothelial cell glycoprotein that plays an integral role in promoting the firm adhesion and transmigration of blood leukocytes (202). Studies examining the adhesive interactions between melanoma and endothelial cells suggest that melanoma cells use their surface very late activation antigen-4 (VLA-4) integrin to adhere to endothelial VCAM-1. Immunohistochemical analyses have revealed that VLA-4 is present in a greater percentage of metastatic melanomas *in situ* than in benign melanocytic lesions (203), and the presence of VLA-4 is negatively associated with disease-free interval and patient survival (204). In a spontaneous murine model of melanoma, VCAM-1 was selectively up-regulated in target organs (brain, heart, and liver) during melanoma metastasis (205), and antibody blockade strategies targeting either VCAM-1 or VLA-4 significantly attenuated the metastatic burden in animal models (206–208).

After arresting in the microcirculation, tumor cells can either grow within the blood vessel or traverse the vessel wall to gain access to the underlying tissue parenchyma. An expansive body

of evidence suggests that platelets play an important role in this process. Platelets have long been regarded as an important accessory cell in the metastatic process because early studies demonstrated that antiplatelet agents could significantly reduce the formation of metastases. For example, Gasic *et al.* (209) reported that the administration of neuraminidase to mice stimulated a thrombocytopenic state and diminished the number of metastases (210, 211). Morphological studies show that platelets aggregate at the tumor cell-endothelial cell junction shortly after cancer cell arrest (212, 213). Several reports have demonstrated a role for the $\alpha_{IIb}\beta_3$ integrin in this process. Normally, $\alpha_{IIb}\beta_3$ is sequestered in platelets and redistributed to the cell surface in response to thrombin (214). Once presented on the platelet surface, the $\alpha_{IIb}\beta_3$ receptor initiates platelet adhesion and aggregation by engaging a number of extracellular matrix components, including fibrinogen, fibronectin, vitronectin, thrombospondin, and von Willebrand factor (215). Aggregated platelets release a variety of mediators, some of which have been shown to augment the expression of integrin receptors on tumor cells. Grossi *et al.* (216) demonstrated that a lipoxigenase metabolite of arachidonic acid, 12(S)-hydroxyperoxyeicosatetraenoic acid [12(S)-HETE], enhanced the expression of $\alpha_{IIb}\beta_3$ on Lewis lung carcinoma cells, resulting in an increase in the adhesion of tumor cells to endothelial cells. Studies have shown that certain tumor cells, such as melanoma cells, also express $\alpha_{IIb}\beta_3$ and use the integrin to recruit platelets and thereby enhance their affinity to the microvascular surface. Chang *et al.* (217) reported that lung-colonizing subpopulations of B16a melanoma cells aggregated platelets to a much greater extent than did B16a cells with low lung-colonizing potential and that this platelet-aggregating potential was correlated with tumor cell expression of $\alpha_{IIb}\beta_3$. 12(S)-HETE has also been shown to enhance the expression of integrin receptors on vascular endothelial cells. Tang *et al.* (218) reported that endothelial cells respond to 12(S)-HETE by up-regulating the vitronectin receptor $\alpha_v\beta_3$ and that this integrin also plays a role in supporting tumor cell-endothelial cell adhesion.

One of the key steps in the pathogenesis of metastasis is endothelial cell retraction, which allows tumor cells access to the underlying basement membrane. Honn *et al.* (219) noted that 12(S)-HETE-producing Lewis lung carcinoma cells induced microvascular endothelial retraction within 15 min and within 60 min had generated the necessary attachments to the subendothelial basement membrane. The endothelial cell retraction was found to be directly related to the tumor cell-derived 12(S)-HETE, because a selective 12-lipoxygenase inhibitor inhibited retraction. These studies support the notion that 12(S)-HETE plays a multifactorial role during the terminal phases of metastasis. First, 12(S)-HETE augments tumor cell adhesion to the endothelium by up-regulating adhesive proteins on both tumor and endothelial cells. 12(S)-HETE then facilitates tumor cell interactions with the basement membrane by stimulating endothelial cell retraction.

E. Lymphatic metastasis

Empirical evidence from clinical reports has produced the impression that the spread of carcinomas takes place primarily through the lymphatic system and that tumors of mesenchymal origins (*i.e.*, melanomas) are more likely to

disseminate through the blood vascular compartment. However, this assumption is difficult to validate because the lymphatic and vascular systems have numerous interconnections (220), and it is well recognized that disseminating tumor cells can pass from one system to another (221–223). Hence, the division of metastatic pathways into lymphatic spread and hematogenous spread is an arbitrary one. Invasive tumor cells can easily penetrate small lymphatic channels and then be transported in the lymph. Tumor emboli may become entrapped in the first lymph node encountered, or they may bypass regional draining lymph nodes to generate distal nodal metastases (“skip metastasis”). Although this phenomenon has been recognized for some time (14), its implications for treatment were largely ignored in the development of surgical approaches for treating cancers (223).

Whether the regional lymph node can trap tumor cells and function as a temporary barrier for further tumor cell spread has been at the center of much debate (224–226). Data from experimental animal systems attempting to address this question have proven difficult to interpret. Many of these studies subjected normal lymph nodes to a single challenge of a large number of tumor cells, a situation that may not accurately pattern the early stages of cancer spread in humans, where small numbers of cancer cells continuously enter the lymphatics (224). Nevertheless, the issue is important because of practical considerations for the surgical management of neoplasms such as cutaneous melanoma (223). The central question concerns whether elective prophylactic lymph node resection can prevent metastasis to visceral organs. The justification for elective lymph node resection in melanoma patients presumes that the metastasis of some lesions occurs first in the regional lymph node and that only later would tumor cells gain access to the systemic circulation to reach distal organs. If this is true, removing the micro-metastases residing in the regional lymph node could clearly increase the cure rate in subgroups of patients with melanoma. There is some evidence in the literature suggesting that the survival rate of patients with melanomas of intermediate thickness (1–4 mm) is improved after elective lymph node resection and that patients with sentinel lymph node disease are more likely to develop distant disease (and die of melanoma) than patients in whom the sentinel lymph node is never positive (227, 228). However, in patients with breast cancer, removal of axillary lymph nodes in a randomized prospective study was not associated with improved survival rates (229).

More recent studies examining the role of the lymphatic system in malignant disease have concentrated on the lymphatic vessels that, among other functions, are responsible for maintaining appropriate interstitial fluid pressure and returning extravasated protein to the bloodstream. Investigations into this area have been facilitated by the identification of a unique set of proteins that are preferentially distributed on lymphatic endothelial cells. The lymphatic endothelial cell hyaluronan receptor, LYVE-1, is expressed on lymphatic endothelial cells (230), liver sinusoidal endothelial cells (231), and macrophages (232), and appears to be the most reliable marker to date for distinguishing lymphatic from blood vascular endothelium. VEGFR-3 is a highly glycosylated tyrosine kinase that is

initially expressed in budding vascular networks but becomes largely restricted to the lymphatic system during later development (233). VEGFR-3 mediates high-affinity binding of VEGF-C and VEGF-D proteins; activation of this signaling cascade has been shown to increase lymphatic endothelial cell division and cell survival (234). In contrast to LYVE-1, VEGFR-3 is also found on proliferating blood vessels (235), so its utility in evaluating tumor-associated lymphatic vessels is limited. Another marker frequently used to identify lymphatic endothelial cells is Prox-1, a homeobox transcription factor that has been shown to function as a key regulator in the differentiation of venular endothelial cells to the lymphatic pathway (236). Prox-1^{-/-} mouse embryos fail to develop lymphatic vessels and die at midgestation (237), and adenoviral expression of Prox-1 in blood endothelial cells has been shown to reprogram these cells into lymphatic endothelial cells (238).

Examinations of the tumor-associated lymphatic vasculature using the aforementioned markers have led to a number of surprising, albeit controversial (239, 240), results. Initial studies reported that overexpression of VEGF-C using recombinant adenovirus could promote lymphangiogenesis, the outgrowth of new lymphatic vessels, in the skin of adult mice (241). Similarly, when tumor cells are genetically engineered to overexpress VEGF-C and VEGF-D, they signal for an increase in lymphatic vessel density that promotes the dissemination of tumor cells to regional lymph nodes (242–247) and more distal tissues (243). Inhibition of VEGFR-3 activation on lymphatic endothelial cells by antibodies or soluble decoy receptors has been shown to attenuate tumor cell metastasis in animal models (246, 248–250). Expression of VEGF-C has been shown to correlate with lymph node metastasis in some human tumors (251–253). Recently, phosphorylation of another lymphatic endothelial cell tyrosine kinase receptor, PDGF-R β , was reported to stimulate the growth of new lymphatic vessels and enhance metastasis (254). Results from our laboratory also support a role for PDGF-R β in lymphatic metastasis in that inhibition of PDGF-R β phosphorylation with imatinib in an orthotopic prostate tumor model resulted in a profound reduction in lymphatic metastasis (255). Additional reports have also implicated VEGFR-2 (256, 257) and hepatocyte growth factor receptor (258) signaling cascades in lymphangiogenesis. These reports suggest that lymphatic endothelial cells respond to receptor tyrosine kinase activation much like endothelial cells from the blood vascular system, a finding that is not surprising given that lymphatic vessels arise from the venous system (237). Although these studies show that an increased number of tumor-associated lymphatic vessels in experimental tumors enhance the probability of metastasis to regional lymph nodes, the contribution (if any) of lymphangiogenesis to the spread of human tumors remains unknown.

Lymphatic vessels provide tumor cells with an avenue to spread to regional lymph nodes and distal organs. Indeed, the importance of the lymphatic system in malignant disease is exemplified by the rigorous clinical assessment of lymphatic tissue for determining tumor stage, prognosis, and

therapeutic intervention (259–262). The bone, lung, and brain are considered target organs of metastasis due to the predilection of tumor cells for these tissues. In the following sections, we discuss the interactions that take place between tumor cells and each of these tissues.

F. Bone metastasis

Current estimates predict that in the United States alone, more than 350,000 individuals die each year with evidence of skeletal metastasis (263). The most common carcinomas to develop bone metastases are those that arise from breast or prostate tumors, with an incidence of 65–75% and 68%, respectively, whereas carcinomas of the lung and kidney metastasize to the bone in approximately 40% of cases (264). The pathophysiology of bone metastasis is complex and involves several different cell populations (tumor cells, osteoblasts, osteoclasts, and endothelial cells) and a number of regulatory proteins (including steroid hormones, cytokines, and growth factors). Traditionally, bone metastases are classified as either osteolytic or osteoblastic, depending on which cell types are involved. The majority of bone metastases arising from the breast are osteolytic in nature, whereas most prostate tumors form osteoblastic lesions in the bone. However, the majority of patients with bone metastases show morphological evidence of both osteolytic and osteoblastic elements (263).

Recent advances in the field of molecular biology produced progress in delineating the cellular and molecular mechanisms responsible for tumor cell metastasis to the bone. For example, the introduction of DNA microarray platforms has provided investigators with a powerful tool with which to identify those genetic determinants that are critical for tumor cell survival in bone. Kang *et al.* (265) recently exploited such an approach to create transcriptional profiles on parental MDA-MB-231 breast cancer and several derivative subpopulations that possessed inherent differences in metastatic potential. These experiments lead to the identification of an underlying gene expression signature in bone-colonizing variants that explained the organ tropism to bone. Compared with the parental MDA-MB-231 tumor population, the bone-colonizing tumor cells expressed significantly more MMP-1, IL-11, osteopontin, connective tissue growth factor, and the chemokine receptor CXCR-4. The coordinated expression of this set of genes explained homing to bone (CXCR-4), proteolysis (MMP-1), angiogenesis (connective tissue growth factor), and osteoclastogenesis (IL-11 and osteopontin).

Bone-derived chemokines such as osteopontin, bone sialoprotein, and stromal-derived factor have been shown to act as chemoattractants for prostate and breast tumor cells and are thought to be responsible for the high specificity of these tumors for skeletal tissue (266–269). Bone-homing tumor cells have a propensity for colonizing the richly vascularized metaphyseal bone found at the ends of long bones, ribs, and vertebrae (270). The cellular interactions that take place between tumor cells and the cells responsible for normal bone homeostasis often result in pathological bone remodeling that results in significant skeletal complications that include pain, hypercalcemia, fractures, spinal cord compression, and immo-

bility. Reports indicate that as many as 80% of patients with stage IV breast cancer have osteolytic bone metastases (271).

A growing body of evidence suggests that one of the primary factors responsible for bone destruction observed during breast cancer metastasis is PTHrP (269, 272, 273). Somewhat surprisingly, reports indicate that expression of PTHrP in primary breast tumors is associated with a more favorable outcome (274). A large prospective study on more than 300 patients with breast cancer determined that patients with PTHrP-positive primary tumors have a more favorable prognosis and significantly fewer metastases to bone and other tissues than those patients whose tumors are PTHrP-negative. However, tumor cell expression of PTHrP is drastically altered by conditions in the bone microenvironment because it has been determined that 90% of breast cancer bone metastases are PTHrP positive (275). Additional evidence implicating PTHrP as a causative factor in the destruction of skeletal tissue comes from preclinical studies demonstrating that neutralizing antibodies directed against PTHrP abrogate osteolytic lesions (276).

PTHrP initiates a vicious cycle of bone destruction by binding to the G protein-coupled PTH receptor that is present on osteoblasts. In normal bone, the activity and generation of osteoclasts is regulated by the ratio between the cytokine receptor activator of nuclear factor κ B ligand (RANKL) and osteoprotegerin, an osteoclastogenesis inhibiting factor (277). PTHrP disrupts this equilibrium by up-regulating RANKL on osteoblasts and decreasing expression of osteoprotegerin, which leads to the differentiation of preosteoclasts and bone resorption by mature osteoblasts (270, 277). Bone resorption liberates TGF- β from the bone matrix, allowing it to bind to its corresponding receptor on tumor cells. Once TGF- β binds to its receptor, a positive feedback loop is activated as TGF- β binding signals for increased tumor cell production of PTHrP (277). PTHrP is also one of the primary factors responsible for the hypercalcemia that is observed in breast cancer patients with advanced bone metastases (278, 279).

Recent studies have demonstrated that the TGF- β released from the degenerating bone matrix may prompt activation of PTHrP-independent osteolytic pathways. Kang *et al.* (280) reported that TGF- β , acting through a Smad-dependent signaling pathway, induces bone-homing breast cancer cells to increase their synthesis and secretion of IL-11, a cytokine with powerful osteolytic activity. As mentioned previously, IL-11 is regarded as a critical determinant in the molecular signature of bone-metastasizing breast carcinoma cells (265). Another member of the IL family, IL-8, has also been shown to stimulate osteoclastogenesis and bone resorption and is characteristically expressed by several types of tumors that metastasize to bone (281, 282). Expression of IL-8 is significantly enhanced by lysophosphatidic acid (LPA), which is a product of activated platelets (283). Boucharaba *et al.* (283) recently reported on the tendency of breast cancer cells to induce platelet aggregation and stimulate the secretion of LPA. Paracrine signaling through the tumor cell LPA type 1 receptor (LPA₁) stimulates not only tumor growth, but also expression of the osteolytic cytokines IL-6 and IL-8. More recently, antagonists to LPA₁ were shown to effectively reduce tumor burden and the accompanying bone destruction

in an experimental breast cancer model (284). These results reinforce the important role that platelets play in the metastatic process.

Cancer of the prostate is the second leading cause of cancer-related deaths and is the most common cancer affecting older men in North America. Mortality from prostate cancer usually results from metastases populated by hormone-refractory cancer cells. To identify factors that may be critical for the growth of prostate cancer cells in the bone, we recently established an orthotopic murine model of hormone-refractory human prostate cancer metastasis to the bone (111). Androgen-independent PC3-MM2 cells were implanted in the bone cortex using a calibrated, push button-controlled device. Five weeks later, we resected the tumor-bearing leg and conducted an extensive immunohistochemical evaluation of these lesions, noting enhanced tumor cell expression of bFGF, VEGF, IL-8, PDGF-BB, and its receptor PDGF-R β . The expression of these proteins was most prominent in lesions growing adjacent to bone. In fact, in tumors that had lysed the bone and extended their growth to the surrounding muscle, we noted only minimal expression of the angiogenic proteins, suggesting that factors in the bone microenvironment were influencing the phenotype of the tumor cells. We also noted that PDGF-R β was activated on both the prostate tumor cells and the tumor-associated endothelium. In contrast, phosphorylated PDGF-R β was not found in either the contralateral nontumor leg or the tumor cells growing in muscle, away from the bone. These results suggested that the PDGF-BB produced by tumor cells acts in an autocrine fashion to stimulate tumor cells and in a paracrine fashion to signal to the tumor-associated endothelium.

The expression pattern of PDGF-R β in the bone metastases suggested that it might be a good target for therapy in that inhibition of this signaling cascade could affect not only the malignant cell population but also the blood vessels that support tumor growth. Indeed, treatment of mice with imatinib or the combination of imatinib plus paclitaxel led to induction of significant apoptosis of both tumor cells and tumor-associated endothelial cells (111). This treatment resulted in smaller tumors, fewer lymphatic metastases, and a significant reduction in bone lysis. These experiments demonstrated that tumor-associated endothelial cells express phosphorylated PDGF-R when confronted with tumor cells that secrete PDGF ligands and that inhibition of this activation, particularly in combination with chemotherapy, can produce a significant therapeutic effect.

To provide a molecular basis for the antiangiogenic effects observed *in vivo*, we generated purified populations of murine bone microvascular endothelial cells (132) and examined their response to stimulation with PDGF-BB ligand and to inhibition of PDGF-R β signaling with imatinib (109). We found that cultured bone endothelial cells expressed PDGF-R β and that the PDGF-BB-induced phosphorylation of this receptor could be inhibited by imatinib in a dose-dependent manner. When bone endothelial cells were stimulated with PDGF-BB, they responded by increasing their cell division, activating the intracellular effectors Akt and MAPK, and up-regulating the antiapoptotic protein Bcl-2. When the bone endothelial cells were subjected to combination treatment with imatinib and paclitaxel, we noted a

significant increase in the number of cells expressing activated caspase-3 and a concomitant decline in Bcl-2 expression. Consistent with these observations, we found that when bone endothelial cells were exposed to both imatinib and low levels of paclitaxel, there was a 3-fold increase in their cytotoxicity. In contrast, treatment of bone endothelial cells with only a single agent produced little effect.

When considered collectively, our data suggested that a primary target for imatinib and paclitaxel might be the blood vessels that perfuse the tumor tissue. We tested this hypothesis by establishing a multidrug resistant prostate tumor cell line by chronically exposing the PC3-MM2 cells to increasing concentrations of paclitaxel (285). The resulting cell line, PC3-MM2-MDR, was 70 times more resistant to paclitaxel *in vitro* than the parental cell line, and its growth in culture was not affected by exposure to paclitaxel or the combination of paclitaxel and imatinib. These data demonstrated that imatinib *per se* does not reverse the resistance of the PC3-MM2-MDR cells to paclitaxel. The bone metastases resulting from injection of PC3-MM2-MDR cells into the tibia of mice produced the same angiogenic profile as that of the parental PC3-MM2 cells. Similar to the bone lesions produced by parental cells, the PC3-MM2-MDR bone lesions were sensitive to the systemic administration of imatinib and paclitaxel. Immunohistochemical examination of these lesions after 14 d of treatment revealed that apoptosis (as measured by terminal dUTP nick-end labeling assay) was largely confined to the tumor-associated endothelial cells, suggesting that the first wave of apoptosis occurs on the tumor-associated vasculature. After 4 wk of treatment with imatinib and paclitaxel, we noted significant apoptosis in both the tumor vascular compartment and the tumor cells. These lesions were characterized by significant necrosis (285). Figure 3 provides a summary of our results generated from the prostate cancer bone metastasis model.

G. Lung metastasis

Results generated from large series of autopsies indicate that the lung is the second most common site for the occurrence of metastasis (286). Among those tumors with a penchant for lung metastasis are those that originate from the breast, bladder, colon, kidney, head and neck, and the skin (melanoma). Treatment options for pulmonary metastases include radiation, chemotherapy, and surgical resection, and successful intervention depends to a large degree on the origin of the tumor. For example, aggressive management (*i.e.*, chemotherapy and metastastectomy) of osteosarcoma patients with pulmonary metastasis results in a 5-yr survival rate of approximately 50% (287, 288), whereas data generated from large studies of patients with melanoma lung metastases typically report a 5-yr survival rate that is below 10% (289).

One distinguishing feature of the lungs that makes it a particularly suitable environment for supporting the outgrowth of metastases is its extremely dense vascular surface area. Indeed, estimates of the vascular surface area in humans indicate that the adult pulmonary vascular bed occupies as much as 100 m² (290), and quantitative measurements of the vascular surface area in rodents show that the lung is severalfold higher than that found in any other tissue (291,

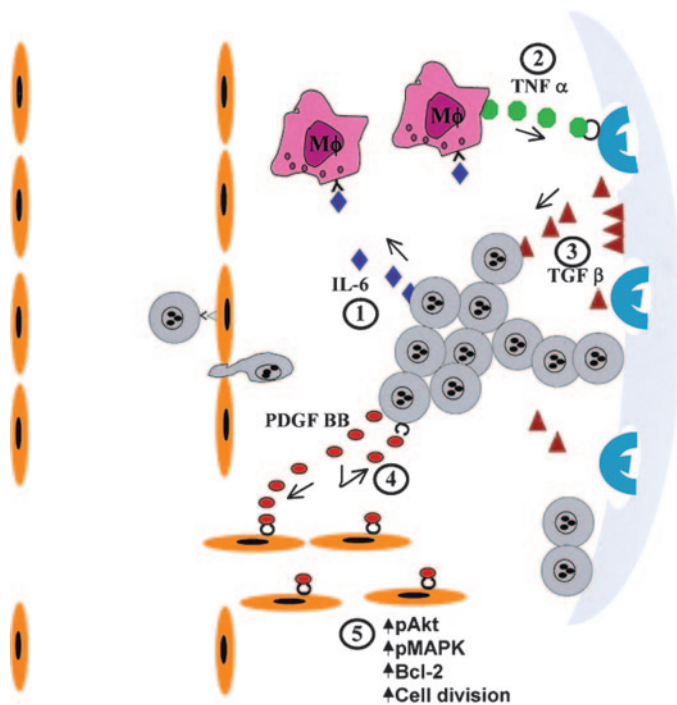


FIG. 3. 1. Prostate tumor cells traffic to the bone through a selectin-dependent process, and once in the bone microenvironment they release IL-6. 2. Tumor cell secretion of IL-6 results in the activation of resident macrophage cells that respond by releasing TNF- α . 3. TNF- α binds to its receptor on osteoclasts, which stimulates bone turnover and the release of TGF- β from the bone matrix. 4. TGF- β binds to the androgen-independent tumor cells initiating the synthesis and secretion of PDGF-BB. 5. PDGF-BB acts in an autocrine manner on tumor cells to increase their proliferation and signals in a paracrine fashion to tumor-associated endothelial cells to promote their proliferation and survival.

292). The lung receives blood flow from two distinct circulations: the bronchial circulation and the pulmonary circulation. Bronchial arteries arise from the thoracic aorta and provide oxygenated blood to the vasa vasorum of the large vessels, visceral pleura, and bronchi to the level of the terminal bronchioles. The pulmonary circulation is responsible for transporting mixed venous blood from the right ventricle to the pulmonary capillaries and returning the oxygenated blood to the left ventricle for systemic distribution. Early studies examining the two different circulations in neoplasms reported that the bronchial circulation supplied blood flow to primary lung tumors (293) and that the pulmonary circulation was more frequently exploited to nourish tumors that had spread to the lung from distal sites (294). However, a recent study has provided evidence that suggests that the pulmonary circulation may also be an important blood supply for some primary tumors (295).

Results generated from experimental models of metastasis in which tumor cells are directly introduced into the venous circulation indicate that pulmonary metastasis is significantly augmented when animals are treated with proinflammatory cytokines before the injection of tumor cells (204, 206). In these studies, the enhanced tumor burden was found to be the result of a direct increase in expression of VCAM-1 on the endothelial cell surface. However, data from sponta-

neous tumor models that require successful completion of all steps of metastasis indicate that primary tumors do not stimulate enhanced expression of VCAM-1 on the lung microvascular endothelium (205). In fact, studies have shown that VCAM-1 expression is actually repressed on the blood vessels perfusing lung metastases in mice (205, 296) and humans (296). Piali *et al.* (296) propose that the down-regulation of VCAM-1 on the tumor-associated vasculature may provide a mechanism whereby metastases circumvent cytotoxic effector cells. Indirect evidence suggests that diminished expression of VCAM-1 observed in metastasis may be due to adhesion molecule shedding. Franzke *et al.* (297) reported that serum levels of soluble VCAM-1 were elevated in patients with malignant melanoma and that this correlated with poor outcome.

Although the aforementioned studies suggest that primary tumors do not augment expression of endothelial cell adhesion molecule in the lung vasculature, there is evidence that suggests that primary neoplasms transmit prometastatic signals to the vascular endothelium before tumor cell dissemination. Indeed, Hiratsuka *et al.* (298) noted that primary Lewis lung carcinomas, B16 melanomas, and a variety of tumors in cancer patients activate VEGFR-1 on distal lung endothelial cells to elicit their synthesis and secretion of MMP-9 before tumor cell spread. These investigators reported that the MMP-9 produced in the lung during the premetastatic phase is critical for the invasion of disseminating tumor cells into this organ. Separate lines of investigation also report a role for VEGFR-1 in preparing the target organ for the arrival of tumor cells. Kaplan *et al.* (299) reported that primary neoplasms release factors that instruct fibroblasts to increase their expression of fibronectin in the lung and other target organs of metastasis. The enhanced expression of fibronectin provides a chemotactic gradient for VEGFR-1 positive hematopoietic progenitor cells that migrate to the lung and form a premetastatic niche. One of the characteristic features of the niche is that it contains abundant amounts of MMP-9 that may release chemotactic factors that provide directional cues for intravasating tumor cells.

Factors produced within the lung environment have also been shown to alter the gene expression patterns of pulmonary metastases in such a manner that the tumor cells are rendered more resistant to the effects of chemotherapy. Wilmanns *et al.* (300) reported that CT-26 colon cancer cells growing in the lungs of syngeneic mice were refractory to systemic administration of doxorubicin, whereas the same cells residing in the skin were sensitive to the drug. The enhanced resistance was attributed to an up-regulation in P-glycoprotein expression in the malignant cells. P-glycoprotein is a member of the ATP-binding cassette superfamily of transporters and a product of the multidrug-resistant gene (301). Functional studies indicate that P-glycoprotein acts as an energy-dependent efflux pump to extrude a broad range of toxic compounds from cells, including several chemotherapeutic drugs (302).

Analysis of data generated from autopsy studies suggests that cancer cells from different types of primary tumors do not spread to all metastatic sites, but rather establish metastases in generalized sites from which further spread occurs (303, 304). Reports have shown that the lung is the generalizing site for cutaneous malignant melanoma in both humans

(303) and mice (305). Alterman *et al.* (305) reported that in spontaneous melanoma models of melanoma metastasis, only mice possessing lung colonies exhibited extrapulmonary metastases. Fidler and Nicolson (306) demonstrated the ability of lung metastases to spread and create tertiary metastases by parabiotically joining tumor-free mice to mice bearing lung metastases. After 2 wk there was no evidence of any tumor growth in the “guest” animals. However, when the parabiont animals were allowed to survive for 4 wk after separation from the metastasis-bearing animals, 40% developed lung metastases. Because the host mice did not have primary tumors at the time of parabiosis, the metastases in the guest mice could have only arisen as metastases from metastases (Fig. 4).

Although much effort has been directed toward that discovery and development of molecular cancer therapeutics, no agent has emerged that possesses dramatic activity for tumors residing in the lung. As previously mentioned, several reports have demonstrated that a characteristic feature of the blood vessels that perfuse several different types of tumors is a tendency to express EGF-R (136–140). In addition, we have reported that the most potent mitogen for lung endothelial cells that were generated from *H-2K^b-tsA58* mice is EGF (134). Based on these observations, one may reach the conclusion that the inhibition of EGF-R on tumor-associated blood vessels may be an effective therapy for tumors in the lung. Unfortunately, clinical trials targeting EGF-R in patient populations with primary lung cancer have, to date, been rather disappointing (307, 308), and this may also prove to be true for tumors that metastasize to the lung. Considering the immense vascular surface area of the lung, it is conceivable that angiogenesis may not be a prerequisite for tumor growth in this organ. Studies conducted on human samples have confirmed that the endothelial cell mitotic index in lung tumors is among the lowest of all tumors and that only 2% of tumor-associated endothelial cells label with proliferation markers (81). More studies on lung tumors are warranted, and it will be important to reconcile whether or not targeting lung endothelial cells is a realistic therapeutic objective.

IV. Tumor Heterogeneity

A. Biological heterogeneity of primary cancers and metastases

Not all of the tumor cells in a primary neoplasm or those that enter the circulation can produce metastases. In fact, less than 0.01% of circulating cells are likely to produce a secondary growth. The development of metastases could therefore represent the fortuitous survival of a few tumor cells or the selection from the heterogeneous parent tumor of a subpopulation of metastatic cells endowed with properties that enhance their survival. Data generated by our research group and many others prove that neoplasms are biologically heterogeneous and that the process of metastasis is indeed selective (309, 310).

In general, investigators have relied upon two approaches to isolate populations of cells that differ from the parent tumor in metastatic capacity. The first approach uses an *in vivo* selection process in which tumor cells are implanted into a given tissue and metastasis is allowed to occur. Metastatic tumors are harvested and the dispersed tumor cells are expanded in cell culture. After multiple rounds of selection, the behavior of the cycled cells is compared with that of the parent tumor to determine whether the selection process enhances metastatic capacity (309) and to confirm that the increased capacity of the cycled cells is not the result of adaptation of tumor cells to preferential growth in a particular organ (311, 312). This approach was originally used to isolate the B16-F10 cell line from wild-type B16 melanoma (309) and has since been employed on a number of occasions to produce tumor cells with enhanced metastatic capacity (313, 314).

In the second approach, tumor cells are selected based on a particular phenotype that is thought to be important in a particular step of the metastatic process. The cells are then tested *in vivo* to determine whether there has been an alteration in metastatic potential as a result of the *in vitro* selection process. The approach has been used to determine whether

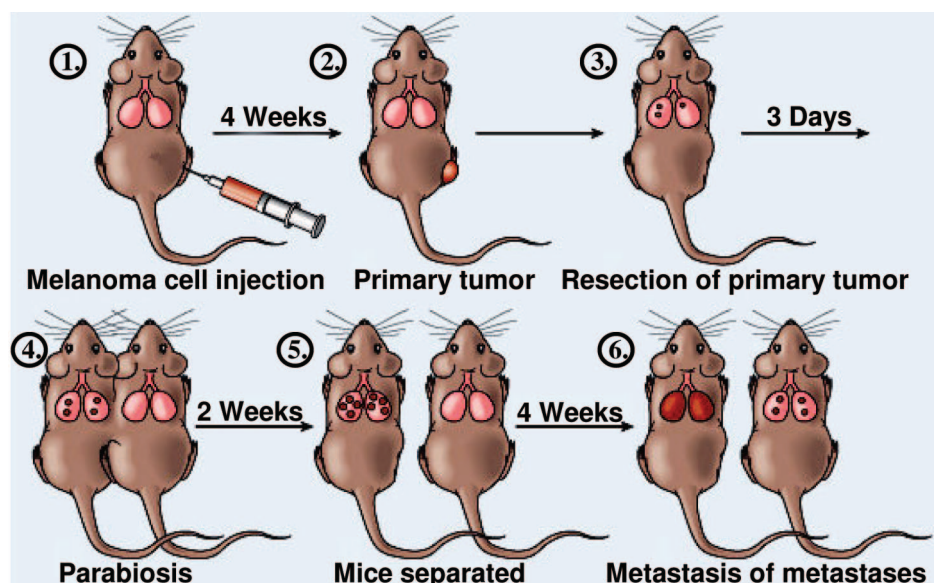


FIG. 4. Metastasis of metastases. B16 melanoma cells were implanted into the footpad of syngeneic C57BL/6 mice. Four weeks later, the hind leg with tumor was amputated, and the tumor-bearing mice were parabiosed to normal syngeneic animals. Two weeks later, the mice were separated. The “guest” mouse developed lung metastases, proving that lung metastases produce additional metastases.

properties such as resistance to T lymphocytes (315), adhesive characteristics (316), invasive capacity (314, 317, 318), and resistance to natural killer cells (319) are crucial for metastasis.

One obvious criticism of these studies is that the surviving isolated tumor cells may have arisen as a result of adaptive, rather than selective, processes. In 1977, Fidler and Kripke (310) provided the first experimental proof of the existence of metastatic heterogeneity in tumors. Using the modified fluctuation assay created by Luria and Delbruck (320), these investigators showed that different tumor clones, each derived from an individual cell from the parent tumor, varied dramatically in their ability to form lung metastases after iv injection into syngeneic mice. Control subcloning procedures were used to demonstrate that the diversity was not a consequence of the cloning procedure (310) (Fig. 5).

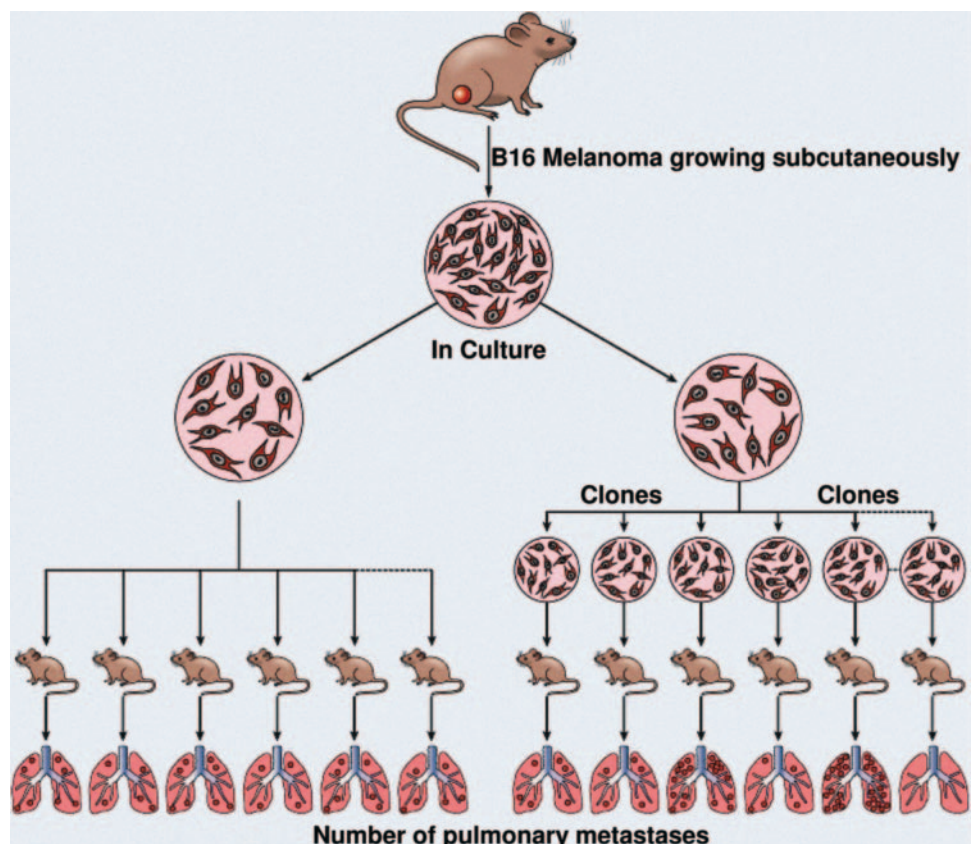
To exclude the possibility that the metastatic heterogeneity observed in the B16 melanoma was the result of lengthy *in vivo* or *in vitro* activation, we studied the biological and metastatic heterogeneity in a mouse melanoma induced in C3H mice by chronic exposure to UV-B radiation and painting with croton oil (321). One mouse thus treated developed a melanoma designated by Kripke as K-1735 (321). The original K-1735 melanoma was established in culture and immediately cloned (322). In an experiment similar in design to the one described for the B16 melanoma (Fig. 5), the clones differed greatly from each other and from the parent tumor in their ability to produce lung metastases. In addition to differences in the number of metastases, we also found significant variability in the size and pigmentation of the me-

tastases. Metastases to the heart, liver, and skin were rarely pigmented, whereas those growing in the brain were uniformly pigmented.

To determine whether the absence of metastasis production by a few clones of K-1735 was a consequence of their immunological rejection by the normal host (323, 324), their metastatic behavior was observed in young nude mice (325, 326). In these mice, the immunological barrier to metastatic cells that also may be highly immunogenic is removed, and the immunogenic cells may successfully complete the process. This was true for cells of two clones that did not produce metastases in normal syngeneic mice but produced tumor foci in the young nude mouse recipients. However, most of the nonmetastatic clones were nonmetastatic in both the normal syngeneic and nude recipients. Therefore, the failure of the clones to metastasize in syngeneic mice was probably not caused by their immunological rejection by the host (324) but rather by their inability to complete one or more steps in the complex metastatic process.

The finding that preexisting tumor cell subpopulations within a primary tumor exhibit heterogeneous metastatic potential has been confirmed using a wide range of experimental animal tumors of various histological origins. Similarly, studies examining human tumors growing in nude mice also identified subpopulations of tumor cells with varying metastatic potential (327–335). It is also clear that cells that survive to form metastases possess a greater metastatic capacity than the majority of cells in an unselected tumor. Examinations with heterogeneous, unselected neoplasms

FIG. 5. Metastasis results from preexisting variant cells within a malignant tumor. B16 melanoma cells growing in cell culture were divided into two parts. One part was injected iv into syngeneic C57BL/6 mice, and the other was used to produce several clones. Once established, clones were also injected into syngeneic mice. Tumor cell suspensions were identical with respect to passage number and number of cells injected. Mice were killed 18 d later, and the number of pulmonary metastases in each mouse was determined (modified from Ref. 310).



concluded that metastasis is a selective process that is regulated by a number of different mechanisms.

B. Clonal origin and development of biological heterogeneity in cancer metastases

Similar to primary neoplasms, experimental evidence suggests that metastases are derived from a unicellular origin. Talmadge *et al.* (336) demonstrated the clonal nature of metastases by using the fact that x-irradiation of tumor cells induces random chromosome breaks and rearrangements. Most of the cell lines cultivated from 21 individual melanoma metastases exhibited unique karyotypic patterns of abnormal marker chromosomes, suggesting that each metastasis originated from a single progenitor cell. Similar results have been reported in other experimental systems (2), indicating that the majority of metastases are clonal in origin. Additional studies have demonstrated that within the population of clonal metastases, variant clones with diverse phenotypes rapidly emerge, leading to the generation of significant cellular diversity within individual metastases (337, 338).

Nowell (37, 339, 340) has suggested that acquired genetic variability within developing clones of tumors, coupled with selection pressures, can result in the emergence of new tumor cell variants that display increasing growth autonomy or malignancy. This hypothesis suggested that accelerating tumor progression toward malignancy is accompanied by increasing genetic instability of the evolving cells. To test this hypothesis, we measured the rates of mutation of paired metastatic and nonmetastatic cloned lines that were isolated from four different murine tumors (341). We noted that highly metastatic cells were phenotypically less stable than their benign counterparts. Moreover, the rate of spontaneous mutation in highly metastatic clones was severalfold higher than in less metastatic clones. Similar results have been reported for other neoplasms (342–344).

Collectively, these studies suggest that the more metastatic a tumor cell population, the greater the likelihood that the cells will undergo rapid phenotypic diversification and thus, be resistant to various therapeutic modalities. In fact, this process may be exaggerated by the mutagenic action of many of the drugs used to treat tumors (345).

V. Conclusions

Despite improvements in diagnosis, general patient care, new surgical techniques, and systemic adjuvant therapies, most deaths from solid cancers are caused by metastases that are resistant to conventional therapies. Because tumor cells are genetically unstable, most primary neoplasms, and especially metastases, are biologically heterogeneous and consist of multiple subpopulations of cells with different phenotypes. The outcome of metastasis is determined by the cross-talk between the “seed and soil”, that is, interactions between specific subpopulations of metastatic cells and host homeostatic factors in specific organ microenvironments that include the vasculature. Clonal metastases can grow progressively in different lymph nodes or in different regions of the same organ where the microenvironment supports the growth and survival of metastatic cells. Understanding the

mechanisms responsible for the development of biological heterogeneity in primary cancers and metastases and the processes that regulate tumor cell dissemination to and proliferation in distant organ tissues is a major goal of research. A new understanding of these issues should lead to the development of therapy against metastases by targeting the metastatic cells and/or the specific organ microenvironment, such as specific vasculature.

Acknowledgments

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