

Review

Progress in Molecular Mechanisms of Tumor Metastasis and Angiogenesis

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Abstract. *The development of metastases is the major cause of death for cancer patients, however, the mechanisms of tumor invasion and acquisition of capability to metastasize remain unclear. During the past decade, knowledge regarding the molecular and cellular processes involved in the regulation of tumor metastases has dramatically increased and has been focussed on cross-talk between selected cancer cells and the specific organ microenvironment. The three-step development of the invasive phenotype of cancer cells is described: cell attachment, local proteolysis and cell migration. The molecular analysis of invasion-associated cellular activities, mainly the role of homotypic and heterotypic cell-cell adhesions, cell-matrix interactions, proteolysis mechanisms and migration properties of cancer cells, are also discussed. The role of tumor phenotype and microenvironment in the metastatic predilection for a specific organ site is pointed out, considering the recent reports which indicate that the capacity to metastasize might be acquired early during multistep tumorigenesis, thereby also predicting the site of metastasis. In addition, this review summarizes the current knowledge regarding angiogenesis regulation in progressive tumor growth and in the complex, multistep nature of tumor cell dissemination. A better understanding of the linkage between genetic and epigenetic events in metastases development may result in new anticancer treatment strategies.*

Biological mechanisms of metastasis development have been studied for more than 100 years, but only recently has the knowledge about this process dramatically increased.

Cancer is malignant because cancer cells invade into neighboring tissue and occupy it. The invasion process permits

neoplastic cells to enter the blood circulation and spread locoregionally with metastases to lymph nodes, or systematically forming secondary tumors in distinct organs. The leading cause of death in cancer patients is not a primary tumor but its metastases, however, it is not clear if all metastases originate from the primary tumors or whether metastases themselves have the capacity to metastasize (1). According to the prevailing model, the ability for metastasis development is a highly selective process, since, among the heterogenous population of a primary tumor, only a small cell subset is able to metastasize (2-5). The formation of metastases is a very complex and dynamic process during which a number of interactions between tumor cells themselves and between tumor cells and the surrounding environment take place (2, 4, 6-11). In oncological language, the steps involved in the process of metastasis are often described as the "metastatic cascade" (2, 10, 12, 13). Initially, single tumor cells or small tumor cell aggregates detach and leave the primary tumor, a process which is called tumor cell dissociation (2). Next, the cells actively infiltrate the surrounding stroma and enter into the circulatory system, traveling to distinct sites to establish the secondary tumor growth (2, 9) (Figure 1). In the bloodstream, a very small number of tumor cells survive to reach the target organ, indicating that metastasis formation must be regarded as a very ineffective event (2, 14). Millions of carcinoma cells enter into the circulatory system, but the majority of them die during transportation, and only 1-5% of viable cells are successful in formation of secondary deposits in distinct sites (2, 5, 14). It is known that many cells of the immune system, such as NK cells, macrophages and lymphocytes, could contribute to the elimination of tumor cells in the vascular system (3, 5). The death of circulating cancer cells may be also caused by very simple factors like mechanical movement, turbulence and lack of proper nutrition and metabolism. In general, the steps required for metastasis development are similar for all carcinomas, and the expression of an invasive phenotype occurs within a dynamic microsystem built by cancer cells, a variety of host cells and the extracellular matrix (ECM) (4, 6, 9).

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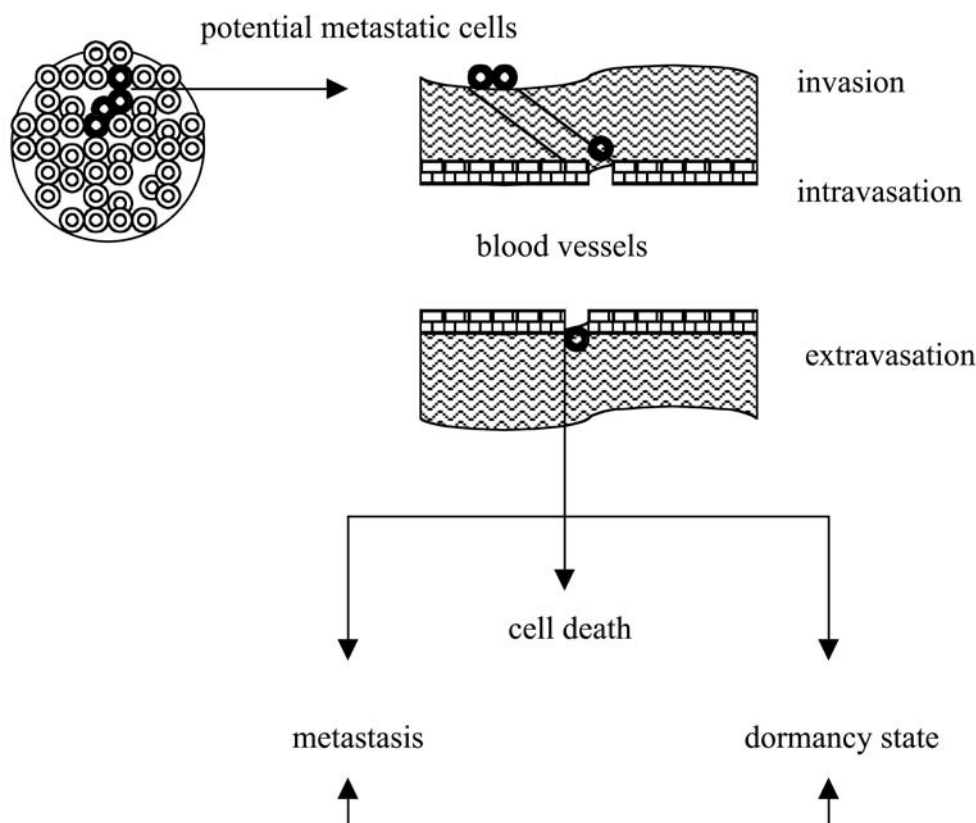


Figure 1. Different steps in the metastatic process of cancer cells.

A malignant tumor is the result of the accumulation of genetic changes in the cell genome leading to loss of normal function, differentiation and sensitivity to death signals. Cancer-related genetic alterations are multiple and appear in varying order, so it is difficult to ascribe the sequence of events, especially characteristic for late stages of tumorigenesis. For a long time, development of the primary tumor and invasion were considered as independently regulated events, supporting the concept of "growth separate from invasion" (10, 12). The growing list of cancer genes, however, comprises several examples of oncogenes and tumor suppressor genes that are implicated in both early and advanced stages of tumor growth (9, 15). The regulatory cascade between the nuclear system and the extracellular environment and survival signals is involved in both primary and secondary tumor growth.

Today, it is known that the potential of tumor cells to metastasize depends on the pre-programmed metastatic capacity of tumor cells (poor prognosis signature) and epigenetic factors provided by the adjacent tumor microenvironment to promote invasion and metastases (2, 9, 14, 16-19).

Cell attachments

In the development of metastases, the existence of at least three essential steps are considered (6, 9, 10, 20): cell attachment, including homotypic and heterotypic cell – cell adhesion, local proteolysis and cell migration. The adhesion molecules, often defined as master molecules, play a crucial role in the metastatic cascade, however, they are not sufficient to explain all steps of this process. Adhesion molecules are responsible for the interactions between cells of the same type (homotypic adhesion), as well as for the attachment of metastasizing tumor cells to extracellular matrix components and to other cells (heterotypic adhesion). On the molecular level, the detachment of single tumor cells or small tumor cell aggregates from the primary tumors requires the loss of homotypic cell – cell adhesion, which is mediated by two main groups of adhesion molecules: the immunoglobulin superfamily and some members of the cadherin family, involving about 30 calcium-dependent transmembrane adhesion molecules (2, 4, 21). E-cadherin has received great attention as a suppressor of invasion and metastasis (2, 6, 9, 10, 12, 21). The extracellular portion of this molecule establishes

adhesion contacts to the E-cadherin of other cells, whereas the intracytoplasmic tail forms a complex with different catenin subunits (α , β , γ), which are involved in signal transduction and gene expression (2, 22, 23). It was found that down-regulation of E-cadherin or defects in catenins are associated with cancer progression (2, 6, 9, 22) and play a key role in the transition from adenoma to colon carcinoma (24). It has also been shown that, in patients with non-small cell lung cancer, undetectable E-cadherin expression indicated shorter overall survival (25).

In a number of human cancers which have lost E-cadherin expression, the switch from E to N-cadherin has been observed (9, 21, 23). N-cadherin presents an opposite effect to E-cadherin and promotes cell motility and migration (21, 23). The detection of its expression is considered as a poor prognostic factor (23, 25). According to published data, the differences between N-cadherin as an invasion promoter and E-cadherin as an invasion suppressor might be explained by differences in modulating intracellular signaling, associated mainly with tyrosine kinase receptors (RTK) (21, 23).

Proteolysis

A very important factor in tumor invasion is the disruption of the basement membrane integrity and the active translocation of neoplastic cells across ECM, after leaving their place of origin (2, 6, 9). This process requires cyclic attachment to, and detachment from, matrix components in a controlled manner, local proteolysis of matrix proteins, pseudopodial extension and cell migration. An important role in invasion and metastasis is played by those proteases expressed by tumor and/or host cells (2, 10, 13), which are divided into the following major groups: matrix metalloproteinases (MMPs), serine proteinases, including the system of plasminogen activators (uPA), cysteine proteinases and aspartate proteinases (2, 6, 9). MMPs represent a family of about 27 enzymes with their central multifunctional role in the proteolytic degradation of all ECM components (26-29). They participate in the release of matrix remodeling enzymes that affect both tumor and microenvironment cells (17). Moreover, some selectivity in action of individual metalloproteinase members has also been considered (26, 29-31). MMPs can be subdivided into distinct categories according to their structural and functional properties as: collagenases, gelatinases, stromelysins *etc.* (6, 30). The role of uPA and MMPs in disruption of some cell adhesion molecules involved in ECM-cell interactions has also been described (30, 32). According to recently published data, the secretion of diverse proteolytic factors is mediated by an inflammatory process observed in many solid tumors and by the infiltration of T lymphocytes, mast cells, neutrophils and

macrophages, causing alteration of local tissue architecture and homeostasis to promote tumorigenesis (17, 33). Metalloproteinases and other types of proteinases may cooperate in the cascade system, however, the relationships between them are largely unknown.

The use of *in vitro* and *in vivo* models has established that the activities of proteolytic enzymes are limited by the action of natural inhibitors such as tissue inhibitors of metalloproteinases (TIMPs) and plasminogen activator inhibitors (PAIs) (6, 13, 26, 29). Therefore, the interplay between the levels of enzymes and their inhibitors seems to be important and responsible for the increase or decrease in the metastatic capability of tumor cells. Remodeling of ECM by proteases and protease inhibitors is accompanied by changes in the composition and organization of the matrix and exposition of cryptic epitopes for different factors activating the promotion of migration, survival and dissemination of malignant cells (11, 32).

Based on these observations, drugs blocking the action of proteolytic enzymes are considered promising targets for cancer therapy (29). An example of such a synthetic metalloproteinase inhibitor is Batimastat (2, 6). A clinical study has recently indicated some inhibitory effect of Batimastat after local application in the treatment of malignant pleural effusions (34), but, in general, the clinical trials of synthetic protease inhibitors have been disappointing (29, 30, 32). MMPs represent just one family of proteinases with matrix degrading ability, and an understanding of the coordinated protease regulation during carcinogenesis seems to be very important for the application of suitable inhibitors for cancer therapy (26, 32).

Cell migration

An important step in the metastatic process is the mobility of tumor cells into the regions of the matrix modified by proteolysis, penetration of extracellular structures, intravasation and extravasation (2, 6, 9, 10, 12, 19, 20, 35). Cellular organs of motility and invasion are termed pseudopodia or invadopodia. Several pieces of evidence indicate that cell surface degradative enzymes and adhesion receptors aggregate at these pseudopodia to control cell migration process (6). In metastatic tumors, increased cell migration is regulated by integrins (32, 36, 37), which act in cooperation with signaling and cytoskeletal proteins, growth factor receptors and regulate the activity of different proteases facilitating the degradation of ECM integrity. The contact of cells with matrix proteins is mediated by integrin molecules, which move the leading cell edge forward, where pseudopodia are protruding, thus contributing to the progress of metastatic dissemination (36).

In stimulation of tumor cell motility, many factors are involved in the molecular machinery for cell locomotion

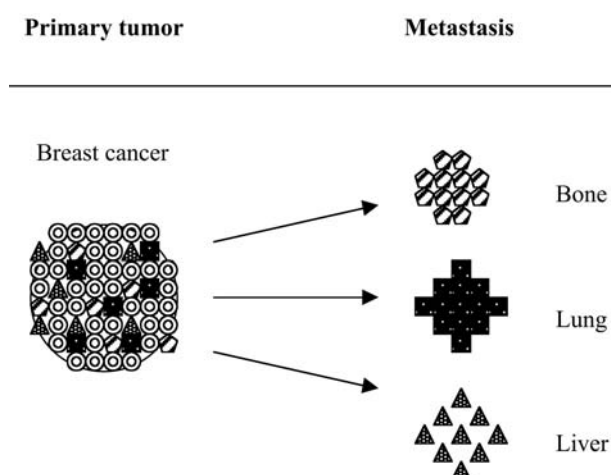


Figure 2. *Poor prognosis signature primary tumor containing cell subpopulations expressing tissue-specific profile, predicting the site of metastasis [adapted from Van't Veer LJ, Weigelt B (16)].*

(10, 20, 25). These factors belong mainly to different growth factor families and pro-inflammatory cytokines and exert both autocrine and paracrine effects in invasion and the metastatic process (6, 9, 17, 19, 23, 35). One of the most important events facilitating the release of chemokines and the dissemination of malignant cells throughout the body seems to be the inflammatory process (17, 32, 38). Some authors have divided the factors regulating tumor-cell migration into three groups (6): autocrine motility factors, soluble matrix proteins acting mainly through integrin receptors and paracrine motility factors secreted by host cells, mainly responsible for the movement of tumor cells toward the organs that produce them. Recently, an important role in the autocrine and paracrine promotion of cell migration has been assigned to the cancer-cell derived cytokine-transforming growth factor β (TGF- β) (23, 36). Its effect implicates stimulation of angiogenesis, escape from immunosurveillance and recruitment of myofibroblasts to produce pro-invasive signals, including the stimulation of proteolytic enzymes (23, 30). According to some reports, in the regulation of tumor cell migration the key factors are neurotransmitters involved in the development of metastasis, as in the case of the well-known chemokine effect (38). An important link between tumor migration and successful metastasis is also the promotion of tumor cell survival (27, 28). It has been shown that, during invasion of the ECM, cells regulate survival mechanisms through the activation of extracellular-regulated kinases (ERK), mainly apoptosis inhibitors. However, the mechanism of this phenomenon remains unknown (39).

Metastatic spread

After passing through the ECM, tumor cells achieve the step which is called intravasation (2). This step also requires well-coordinated proteolysis and locomotion. In the vasculature, tumor cells are passively disseminated by the blood stream to reach the organ for their secondary growth. The majority of metastases develop in the first capillary bed after leaving the primary tumor, indicating that the distribution of metastases is, at least in part, mediated by regional angiogenesis (6, 19). However, tumor cells frequently colonize distant organs (6, 9, 19). The anatomy of the circulation system can explain, for example, the frequent localization of liver metastasis in colorectal carcinoma patients, but the high proportion of bone metastases in breast, lung and prostate cancers is more difficult to account for (6, 9).

The precise mechanisms determining such organ-specific predilection for metastasis remains unclear, however, the majority of authors have pointed out that metastasis development in particular organs is a consequence of the interactions between tumor cells and the supportive role of the environment (6, 9, 19, 35). The organ specificity of metastasis may be promoted by the existence of a special molecular addressing code and a relationship between the adhesion molecules and their receptors on metastatic cells and the cells of the preferred organ (6). It has also been reported that the mechanism for preferential metastasis to specific organs is the selective chemotaxis of circulating tumor cells to the source of the appropriate chemokines (6, 9, 19, 35).

Recent studies, performed on cell lines and human breast cancers, have indicated that the capacity to metastasize might be acquired early during multistep tumorigenesis and is displayed by the whole tumor cell population (16, 40). This means that the disease outcome of breast cancer patients could be predicted by a "good" or "poor" prognosis signature of the primary tumor (16, 40-42). Some months ago, Wang *et al.* (43) testing, patients with lymph-node-negative primary breast cancer, found 76 gene signatures which belong to many functional classes and appeared to be highly informative in identifying the patients who developed distant metastases within 5 years. Other reports indicate that subpopulations of poor prognosis signature tumors showed a tissue-specific expression profile, which predicted the site of metastasis development (16, 40) (Figure 2). It has also been shown that gene expression profiles of primary breast cancers and their metastases are comparable (18). These findings clearly confirm that metastatic phenotypes appear early in tumor progression (16) and suggest that knowledge of the gene set expression of the primary tumor is a rational base for micrometastasis therapy (18).

Tumor dormancy

From clinical observations it is known that distinct metastasis may occur even many years after the removal or successful therapy of the primary tumor (2, 19, 44), indicating that malignant cells after extravasation are able to remain dormant, but viable, for some period of time (2, 19, 44, 45). The phenomenon of tumor dormancy has received little attention, however, it is evident that many extravasated cells, as a consequence of some shift in tumor-host interactions, are capable of expressing their malignant potency and establishing distinct metastases even many years after successful removal of the primary tumor (2, 19, 44). Today, the maintenance or induction of tumor dormancy seems to represent an attractive approach for the prevention of both tumor relapse and the formation of distant metastases.

Angiogenesis

By definition, tumor metastasis involves the transport of cancer cells through the vascular system. The blood and lymphatic vessels participate in all steps of the "metastatic cascade" including: primary tumor growth, stimulation of local invasion, traffic of malignant cells and development of distant metastases (45). It is known that, without blood, vessels tumors can not grow beyond 1-2 mm (44, 46, 47). Highly vascular tumors show a higher potency to produce metastases compared to less angiogenic tumors (3, 45, 48-50). Angiogenesis is mediated by multiple angiogenic molecules released by both tumor and host cells (17, 45, 46, 48, 51). The degradation and alteration of the ECM architecture by the release of different proteases also contributes to the formation of a tumor-associated vasculature favoring tumor cell dissemination (11, 17, 52). The most important angiogenic factors are the members of the fibroblast growth factor, vascular endothelial growth factor and angiopoietin families (47, 48, 50, 53-55). Recently, a potential metastasis-associated gene and its product, the metastatic tumor antigen 1 (MTA1), have been identified. The expression of this antigen appears to correlate with large tumor size and vascular invasion in patients with hepatocellular carcinoma (56). The intensity of angiogenesis is considered to be a prognostic marker and a predictor of cancer relapse (46, 48, 53, 55). Our own studies have revealed that high VEGF concentrations in sera and ascitic fluids of patients with ovarian carcinoma determined significantly shorter overall survival (53). Unfortunately, the prognostic significance of ascitic VEGF levels was lost in multivariate analysis, which revealed that only FIGO stage, age of patient and serum VEGF concentrations were independent prognostic factors for overall survival in ovarian carcinoma patients. It has also been postulated that VEGF participates in the stimulation

of both migration and survival of malignant cells, albeit by distinct signaling pathways (32).

An important indicator of tumor progression is also the lymphatic system. It is now apparent that different members of the VEGF/VEGFR family are involved in lymphangiogenesis (45, 55). Recently, VEGF-C has been considered as a factor which induces selective growth of lymphatic vessels (45, 55) and a correlation between VEGF-C lymphoangiogenesis and lymph node metastasis for many tumors including prostate, colorectal and lung cancers has been found (45, 57). Furthermore, vascular permeability and angiogenesis depend on tumor type and host organ (51).

Tumor survival and metastasis are controlled by the balance between angiogenesis stimulators and inhibitors (45, 46, 48, 51). Inhibitor factors and angiogenic stimulators are produced by host and tumor cells, and their activity is dependent on tumor location (51). At present a large, diverse family of angiogenesis inhibitors is known including angiostatin, endostatin, vasculostatin, tumstatin *etc.* (11, 32, 44, 47, 51, 55), however, further studies are needed to explain the complex cooperation between pro- and anti-angiogenic molecules. The balance between these factors seems to be one of the key determinants influencing tumor cell behavior (11). Novel strategies for inhibiting angiogenesis are currently being developed and include the application of anti-VEGF monoclonal antibodies (55, 58). It was very recently reported that even a single infusion of the VEGF-specific antibody, bevacizumab, showed a direct and rapid antivascular effect in human rectal cancer (58). Similarly, some synthetic molecules, such as GFA-116, have been found to selectively inhibit VEGF-dependent signaling and to suppress angiogenesis and tumorigenesis (59). Functionally, any tumor in which the level of anti-angiogenic factors exceeded the level of pro-angiogenic factors would be dormant and thus undetectable (44, 51). Theoretically, the maintenance of cancer cells in the dormant state should prevent both tumor recurrence and the formation of clinically apparent metastases (2). However, it is possible that angiogenesis in primary tumors and metastases are mediated by different mechanisms, and the response to the same therapy may be not equal (45). Preliminary results from early phases of clinical trials with anti-angiogenic therapy suggest that the best results were obtained with low doses of inhibitors applied long-term, taking into account that the endpoint of anti-angiogenic therapy is not complete tumor elimination but reduction in size sufficient to be independent from increased vascularity (46, 48, 51). Anti-angiogenic treatment was neither toxic nor affected by drug resistance, an unresolved problem in conventional chemotherapy (45, 46, 55), however, angiogenesis is only one step in the metastatic cascade which is necessary, but not sufficient, for metastasis development.

Research into tumor angiogenesis has clearly revealed the importance of the tumor microenvironment in disease progression and response to therapy (47). The basic goals for the future are to more precisely determine the molecular mechanisms which regulate angiogenesis at each step of the metastatic cascade and to evaluate the role of anti-angiogenic factors in detail, taking into account the possibility of administering a cocktail of different inhibitors for more effective cancer therapy (51, 55).

Conclusion and Perspectives

The complex and complicated metastatic process is considered to be the result of interactions between the intrinsic properties of cancer cells and various epigenetic influences, that ultimately control all steps of the metastatic cascade. The current state of knowledge indicates that metastatic disease is a function of the genetic "make-up" of cancer cell populations and of epigenetic events, which act like an expanding spiral in the development of terminal disease (45). Tumors with a high intrinsic capacity to metastasize, recently termed "poor prognosis signature tumors" subsequently form the secondary growth in preferred organs, in a way described by Stephan Paget (60) more than 100 years ago, as the "path of seed and soil". Clarification of the mechanisms responsible for the organ specificity of metastasis and an understanding of the multifactorial nature of tumor progression, especially considering the complex and dynamic interactions between malignant cells, proteases and ECM proteins, are the greatest challenges in oncology. Research in this area will facilitate the development of new, appropriate therapeutic anticancer strategies.

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