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Mechanisms of tumor invasion and metastasis

J. A. Lawrence, P. S. Steeg

Women's Cancers Section, Building 10, Room 2A33, Laboratory of Pathology, National Cancer Institute, Bethesda, MD 20892, USA

Summary. Metastasis is the cascade of events involved in the transition of a malignancy from a localized tumor to the establishment of a distant foci. In this review we define the known stages and the factors involved. The multiplicity of steps involved allows many opportunities for therapeutic intervention. Current research has focused on interruption of a step or a series of steps to limit the spread of cancer. With emphasis on the urologic malignancies, we outline the research thus far accomplished in the field of metastasis.

The metastatic cascade is defined by a complex series of steps initiated by tumor-cell motility, adherence, and proteolysis leading to invasion. Intravasation and extravasation from the circulation are followed by angiogenesis and colonization. The complexity of the events that are achieved by a cancer cell against a host defense system is remarkable. The study of metastasis from all facets has led to an improved understanding of the degrees of lethality of different carcinomas. A commonality throughout the study of metastasis has been a tightly regulated balance of positive and negative influences [38] that maintains the host cellular microenvironment and the discovery of a cancer cell's exploitation of these local factors to invade and colonize more effectively.

Many of the hypotheses that have been proposed and tested in the laboratory have been derived from direct clinical observation. For example, the observation of a hypercoagulable state in a cancer patient led to an inquiry into the role of the fibrinolytic system [19, 70]. The surgical observation of tumor vascularity highlighted the importance of angiogenesis [10]. Additionally, the pathologic observation of the prognostic importance of the integrity of the basement membrane (BM) [1] led to its extensive study.

An obvious attraction to the study of metastasis is the clinical benefits to be gained from an interruption of one step or a series of steps in the malignant process. Effective interventions in metastasis not only impact on localized disease but may also be significant to the treatment of late-stage disease, since the initial events have been completed by the time of cancer detection. The significance of local regional spread of disease is exemplified in renal cancer, in which local spread of disease to the regional lymph nodes dramatically changes the overall survival. According to National Cancer Institute SEER data for the 1973–1991 interval, spread to regional lymph nodes depreciated the 5-year survival of patients with renal cancer from 87% for localized disease to 57% for regional disease [54]. In contrast, in prostate cancer, surgery or radiotherapy can be effective for the treatment of regional disease, but effective therapy is needed for metastatic disease. Again, this is reflected in the SEER data in that the 5-year survival of patients with distant disease is 29% as compared with 85% for regional disease. Although the data suggest that interruption of the metastatic cascade would be of clinical benefit to urologic cancer patients, not all parts of the metastatic cascade may be equally rewarding. For patients with true localized disease, therapy aimed at proteolysis or tumor-cell motility may be more relevant; if micrometastases are present, inhibition of angiogenesis and colonization may be more effective.

Correspondence to: J. A. Lawrence, Fax: 301-402-8910

Cell adhesion

Adhesive interactions between like cells (homophilic), different cell types (heterophilic), or cells and the extracellular matrix (ECM) exert multiple influences on the metastatic process. Issues of current interest in urologic and other malignancies concern the redundancy of tumor-cell adhesive interactions, their functional importance, and the emerging importance of endothelial cell adhesive interactions in angiogenesis.

Traditionally, four broad classes of cell-adhesion molecules (CAMs) have been defined. The first major group is the cadherins, which are calcium ion-dependent and mediate cell binding. Specifically, they mediate homophilic interactions such that cells expressing different cadherins separate from each other and aggregate with cells expressing a similar cadherin [27]. The second major class of CAM is the immunoglobin superfamily, which is a larger and more heterogeneous group that mediates both heterophilic and homophilic interactions. The members of this class are expressed on many types of cells, including lymphocytes, where they play a role in the interaction of T-cells with antigen-presenting cells. A third major class is the selectins, with only three known members. Selectins

are expressed on blood cells and endothelial cells and are involved in heterophilic interactions; thus, they play a role in leukocyte adhesion [27]. The fourth class, the integrins, have received considerable attention in metastasis study because members of this class mediate interactions with the ECM [13]. The integrins are a complex of α- and β-subunits; 14 α-subunits and 8 β-subunits have been described, giving rise to 100 potential combinations, most of which bind multiple ECM proteins. The integrins interact extracellularly with ECM components and intracellularly with the cytoskeletal proteins actin, talin, and μ-actinin [543]. The first integrin-binding site identified on the ECM protein fibronectin was the tripeptide Arg-Gly-Asp (RGD) [59], and a host of other binding sites have since been identified.

Expression and functional data have contributed to the hypothesis that E-cadherins mediate homophilic cellular interactions and that loss of E-cadherin expression may contribute to cell detachment, dedifferentiation, and/or invasion in cancer progression. Decreased E-cadherin expression was correlated with increased aggressiveness in the Dunning-rat prostatic carcinoma model but not in the aFGF (acidic fibroblast growth factor)-induced spread of rat NBT-II bladder carcinoma cells [6]. In human tumor cohorts, decreased E-cadherin expression was associated with decreased 5-year survival in renal carcinoma [35], low risk in bladder carcinoma [51], and poor cellular differentiation in prostatic carcinoma [71]. Functional studies using anti-E-cadherin antibodies or transfections have demonstrated the direct involvement of E-cadherin in several of these systems. E-cadherin cDNA was transfected into dog kidney and mouse mammary tumor-cell lines; those with high expression exhibited a relative decrease in invasion as compared with the control transfectants [73]. When used to treat a differentiated epithelial cell line, epithelial Madin-Darby canine kidney (MDCK), a monoclonal antibody to E-cadherin caused both cellular transformation and in vitro invasiveness on collagen and embryonal heart tissue [3].

Not all CAMs function similarly. Using a panel of cell lines established from renal-cell carcinomas, Zocchi et al. [84] observed that increased N-CAM expression was correlated with increased growth rate, loss of adherence to inert substrate, and increased binding to endothelial cell heparan sulfate, an ECM component. Transfection of COS7 renal cells with N-CAM resulted in increased cellular binding to the endothelium and to heparan sulfate, and it may potentiate growth-factor signaling.

The functional importance of integrins to tumor-cell binding and subsequent cell signaling to ECM has been widely reported. Additional factors that contribute to this interaction are the multiplicity of integrins found on tumor cells and the production and integrity of ECM in the tumor-cell microenvironment. For instance, normal cells secrete ECM components around themselves, but in vitro studies have shown that tumor cells produce less ECM, if any, and rely on tumor-cell surface integrin interaction with the host ECM components [58]. Most cells express an assortment of integrins; among four human prostatic carcinoma cell lines, the α3-, α5-, α6-, β1-, β3-, and β4-subunits were expressed [81]. This level of complexity has been well described in a study using the human PC-3 prostatic carcinoma cell line [14]. Serial invasion of PC-3 cells through a reconstituted basement membrane produced a stably more invasive subline, I-PC-3. I-PC-3 cells attached to multiple ECM components as well as did PC-3 cells but failed to spread on any of the components when used as a matrix. The I-PC-3 cells also exhibited decreased production of fibronectin and a preferential expression of the α6β4 integrin. A functional demonstration of integrin involvement in metastasis was reported in bladder carcinoma, where antibody to the β1 integrin chain inhibited T24-cell invasion through a basement membrane extract [24].

An emerging theme is the involvement of integrins in endothelial adhesion in both metastasis and angiogenesis. Recent reports have demonstrated the involvement of the αvβ3 integrin in angiogenesis [8]. Among urologic carcinomas the α4β1 integrin was hypothesized to mediate heterophilic interactions between the tumor and VCAM-1 on endothelial cells. Increased α4β1 expression was observed on metastatic renal-cell carcinomas, and antibody to either α4β1 or VCAM-1 disrupted renal-cell carcinoma adhesion to human umbilical-vein endothelial cells (HUVEC) [69].

Nontraditional CAMs have also been described and may contribute to the metastatic cascade. For instance, metastatic rat prostatic carcinoma cells bound lung endothelial cell vesicles via dipeptidyl peptidase IV [33], which is thought to be a fibronectin-binding protein. Pienta et al. [53] concentrated on the carbohydrate-binding proteins on the cell surface that interact with the ECM. They reported that modified citrus pectin, which interferes with specific carbohydrate-binding molecules, inhibited the metastatic potential in vivo and adhesion to endothelial cells in vitro of rat prostatic carcinoma cells.

Proteases and motility

Protease remodeling of the tumor microenvironment is a key step in the invasion process. Although a substantial variety of proteases have been identified, basic research questions remain concerning their cellular sites of production and mechanism of action.

The plasminogen-activator system has been implicated in clot formation, interaction of cells with the endothelium, and tumor invasion. The proenzyme plasminogen is involved in the final steps of the extrinsic fibrinolytic pathway. Plasminogen is converted to plasmin by one of two different serine proteases, tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA), and the activated plasmin then degrades fibrin and fibrinogen. A localized and tightly regulated system contributes to overall enzymatic activity [4], typical of several types of proteases. Secreted pro-uPA binds to a uPAR (uPA receptor) on the same cell or a neighboring cell. Enzyme bound at the cell surface is activated to cleave plasminogen and procollagenase IV unless it is inhibited by the binding of PA inhibitor 1 (PAI1), PAI2, or nexin 1 inhibitors. Once PAI1 is bound to the uPA:uPAR complex, the receptor is internalized and its ligand is degraded in intracellular lysosomes and recycled to the cell surface; such trafficking may permit repositioning of uPAR to change the area

of localized proteolysis. The importance of the uPA system to tumor invasion was demonstrated by studies in which basement membrane invasion by tumor cells was blocked in vitro by antibodies to the uPAR [50]. A chloramphenicol acetyltransferase (CAT)-expressing prostate-cancer cell line (PC-3) was transfected with mutant uPA (Ser356→Ala) as compared with control, wild-type uPA, The metastatic potential in the mutant uPA group was reduced, while the primary tumor sites were equivalent. The CAT activity was then measured at the expected sites of metastasis: regional lymph nodes, brain, and lung. The control transfectants showed CAT activity in all lymph nodes, whereas in the mutant transfectants the activity was at background levels [11].

Another important class of proteolytic enzymes is the zinc metalloproteinases, which are found in greater abundance in the more invasive phenotypes of many tumor types, including prostate cancer. The family of zinc metalloproteinases share several properties, including secretion as a proenzyme and requirement of cleavage for activation and inhibition by metal chelators and by tissue inhibitors of metalloproteinases (TIMPs), analogous to the previously described system. There are 11 members of the group, including the fibroblast and neutrophil collagenase, collagenase III, metalloelastase, MT-MMP, stromelysin-1, stromelysin-2, matrilysin, gelatinase A, and gelatinase B, all of which differ in their selective substrate within the BM [22, 65]. The enzymatic activity is pericellular and tightly regulated, although the nature of the cellular receptors and endogenous activators are incompletely defined. Extensive investigation has been reported on the tissue inhibitors of the matrix metalloproteases TIMP-1, TIMP-2, and TIMP-3 [72] because of their obvious therapeutic implications. All three TIMPs have been sequenced from human cells and their functional domains characterized. They bind tightly to the active forms of the metalloproteinases and also with the proenzyme form of gelatinase A and gelatinase B, although the specificity varies for each TIMP. The effective extent of proteolysis is dependent on the local concentration of metalloproteinases versus TIMPs [12, 17, 64].

Several types of chemotactic responses occur in tumor cells. Random motility is observed under conditions lacking a chemotactic gradient. Many growth factors and motility factors elicit directed chemotactic activity, observable in the presence of a concentration gradient, whereas tumor cells can also mount directed haptotactic responses to ECM components. In vitro analysis of cell motility is typically assessed by the use of a Boyden chamber, a multiwelled two-compartment chamber separated by a filter containing pores that are smaller than an average cell diameter. A more sophisticated development in motility study is the use of time-lapse video microscopy to look for indicators of cell motility such as cell membrane ruffling, pseudopod formation, and translocation. The above-mentioned parameters were used for the prostate Dunning cell lines and correlated with the known metastatic potential of the sublines [55]. Of the compounds known to have chemotactic activity, increased autocrine motility factor expression in concert with loss of E-cadherin expression was reported to identify poor-prognosis bladder carcinomas [51], and scatter factor/hepatocyte growth factor induced motile and invasive behavior in NBT-II bladder carcinoma cells [2].

Anoikis

The composite epithelial cell:ECM interaction, including adhesive events and the effects of proteolytic remodeling, have recently been postulated to impact cellular phenotypes other than invasion. Frisch and Francis [23] noted that disruption of epithelial cell interaction with the ECM induced apoptosis, called anoikis. Resistance to MDCK-cell anoikis was achieved by oncogene transformation or treatment with scatter factor. Although not similarly named, a recent elegant study by Witty et al. [82] found that, in contrast to expectations, stromelysin-1 transgeneic mice showed no evidence of increased invasion or metastasis but instead exhibited reduced tumor formation. The disruption of tumor cell:ECM interactions by high levels of protease, leading to apoptosis, was thought to be causal. These data indicate the need to evaluate a wide range of phenotypes in the consideration of potential therapeutic strategies, such as protease inhibitors for control of invasion.

Angiogenesis

Angiogenesis, the development of new capillaries from preexisting blood vessels, is required in normal situations such as wound healing and development but is critical to the continued growth of both primary and metastatic tumor deposits. The angiogenic process is complex, resembling tumor-cell invasion in certain characteristics [38]. Typically, the basement membrane is dissolved at a post-capillary venule, after which endothelial cells proliferate near the venule and migrate toward the tumor. Canalization, branching, and formation of vascular loops complete a functional connection. Progressive arteriolization is also observed in tumor angiogenesis, in which smooth-muscle cells proliferate and encase a developing artery.

The vasculature of the tumor bed has a unique, poorly organized composition. As determined by histologic observation, the architecture has the easily recognized components of arterioles, capillaries, and venules; however, blood channels have been observed in melanomas and sarcomas that are not lined by endothelial cells, the blood therefore coming in direct contact with the tumor [75]. Tumor cells may also be found within the endothelial lining [26]. The postcapillary venules are devoid of BM and are a recognized site of cancer-cell intravasation [32, 76]. In comparison with the normal circulatory architecture, the venous system outnumbers the arterial system, and the pressure in the venules is disproportionately lower than that in the arterial system. This combined with the increased interstitial pressure in the tumor bed, results in a more sluggish circulation and, hence, in a tendency toward vessel-wall collapse [31].

One of the most profound influences on the angiogenic process is the endothelial cell interaction with the ECM.

As an endothelial cell migrates across the ECM it undergoes a change in morphology from a cuboidal to an elongated form. The composition of the ECM is contributory to morphology, proliferation, and differentiation. In vitro studies have demonstrated that endothelial cell culture on BM components induces relatively rapid tube formation, whereas culture on interstitial collagens results in proliferation [42, 43]. The signaling for these processes is dependent on the ECM components and the interaction of the CAMS, but the contact appears to be a mechanicophysical property that relays to the intracellular cytoskeleton proteins [29].

Another regulatory point for angiogenesis is the presence of angiogenic factors. A plethora of such factors have been described as reviewed elsewhere [5], including the FGFs, vascular endothelial growth factor (VEGF), transforming growth factors (TGFs), tumor necrosis factors (TNFs), platelet-derived endothelial growth factor, angiogenin, and epidermal growth factor (EGF), among others. This redundancy may represent a major problem with the development of effective angiostatic agents, as the development of an inhibitor to one class of angiogenic factors may be offset by tumor-cell production of another.

Tumor vascularization may also reflect the degree of activity of angiogenic inhibitors as well as stimulators [38]. Endogenous inhibitors such as the ECM component thrombospondin (TSP), cartilage-derived inhibitor, TIMPs, platelet factor 4, and interferons (IFNs) may act by multiple mechanisms.

Evidence that angiogenesis is a critical component of metastatic spread and patients' prognosis has derived from several approaches, one of which is the correlation of immunohistochemically detected tumor microvessel density with tumor histopathologic criteria or patients' clinical course. These studies, first performed in breast carcinomas [77], have been extended to several urologic malignancies. The most complete characterization has been reported in prostate carcinoma, distinguishing tumors of grade C from those of grade D [21], tumors with capsular penetration and/or pelvic lymph node involvement [7], and tumors with high Gleason's scores and clinical metastases [78]. In an interesting study of clinically localized prostatic carcinoma treated by radiotherapy, microvessel density correlated with pretreatment prognostic factors and 4-year outcome [25]. Similar studies in bladder carcinoma correlated high microvessel density with a 2.5-fold-increased risk of mortality [16]. These studies have not yet established this approach as an independent prognostic factor; thus, larger confirmatory studies are required. Of the many angiogenic factors that could potentially fuel the outgrowth of urologic primary and secondary tumors, several have been implicated. These include FGF [47], VEGF in bladder and renal carcinoma, platelet-derived endothelial-cell growth factor [48, 60, 67], and TGFs in prostatic carcinoma [63]. These studies emphasize the redundancy of angiogenic stimuli and the potential difficulties involved in the development of therapeutic approaches.

Several translational approaches to angiogenesis have been developed. The clinical phenomenon of a rapid increase in metastasis after surgical resection of a tumor launched a search for a tumor-produced inhibitor to angiogenesis [66]. A potential explanation for this is that angiogenesis-stimulating factors are produced by the primary tumor in excess of inhibitors. At the primary tumor site the stimulatory effect is greater and neovascularization occurs, but distally, systemically released inhibitor suppresses neovascularization at metastatic foci. The proposal led to a series of experiments that uncovered a novel angiogenesis inhibitor, angiostatin, that was isolated from the urine of mice with metastasis from the Lewis lung carcinoma low metastatic variant (LLC-LM). The serum and urine of these mice inhibited endothelial proliferation in vitro, and protein purification has identified a 38-kDa protein that shares homology with plasminogen, although intact plasminogen has no angiogenically inhibitory effect [49]. Other endogenous inhibitors of angiogenesis such as the IFNs, TIMPs, and TSP stand as candidates for translational development [79, 80]. Another translational approach was based on the hypothesis that many angiogenic factors are heparan-binding growth factors. Compounds such as pentosan polysulfate have been reported to inhibit angiogenesis in vitro, possibly through heparan-binding growth factors, with resultant inhibition of Dunning-rat prostate carcinoma growth in vivo at the earliest stages [46]. The antibiotic fumagillin and derivatives, including TNP-470, have demonstrated antiangiogenic activity and are thought to target angiogenic factors through non-heparan-binding mechanisms [30, 45, 83]. Another exciting approach to antiangiogenesis was recently reported in the Dunning-rat prostatic carcinoma system by Vukanovic and Isaacs [74]. The immunomodulator linomide was likely inhibitory indirectly via suppression of tumor-associated macrophage activation and inhibition of the elaboration of angiogenic factors.

Colonization

The outgrowth of tumor cells at distant sites remains one of the steps in the metastatic cascade that are "open" for therapeutic development in a large number of cancer patients. Research principally conducted in melanoma has developed the hypothesis that the colonization response of metastatically competent tumor cells is qualitatively different from that of nonmetastatic tumor cells. This difference centers on the production of and responsiveness to growth factors present either in the microenvironment or in the circulation, leading to a more aggressive metastatic phenotype. Examples of "clonally dominant" colonization from metastatic melanoma include a decreased requirement for exogenous growth factors in vitro and autocrine production of certain growth factors [61]. Furthermore, using several different tumor-cell types, Kerbel et al. (reviewed in [40]) and other investigators [9, 39, 41, 57] have reported varying degrees of altered responsiveness to traditionally inhibitory cytokines such as TGF-β, interleukin 6 (IL6), and oncostatin M. For example, TGF-β is inhibitory to the colonization of many nonmetastatic cells but either lacks inhibitory activity or is actually stimulatory for metastatic tumor cells. Little work has been reported on colonization in models of urologic tumor metastasis. The signal-transduction process, whereby a metasta-

tically competent cell can turn an inhibitory signal into a stimulatory one, may represent a very important target for translational investigation in a wide variety of tumors.

Metastatic competence

An intriguing question is raised throughout the metastasis literature: what events control the metastatic cascade? Many attempts at finding the instigating factors have relied on comparisons of metastatic with non-metastatic tumor lines using subtractive hybridizations, differential display, or similar techniques. More recently, chromosomal transfer and walking techniques have been successfully employed. Transfection or transduction experiments remain the means of proving that a particular gene has metastasis-regulatory activity. Necessary features of these experiments include side-by-side control transfectants and in vivo analysis of tumor metastatic potential, particularly by injection of tumor cells at an orthotopic site. One difficulty with these approaches is that, in contrast to the relative clarity of data obtained using a model system or tumor-cell line, the significance of a unique marker is often confounded in clinical cohort studies by the complex and heterogeneous phenotypes encountered.

Cohort and transfection studies have indicated a role for the p53 suppressor gene in the regulation of the metastatic cascade for some urologic tumors. An interesting prostatic carcinoma case was reported by Effert et al. [20], in which intratumoral heterogeneity was observed in p53 in the primary tumor, which contained both wild-type- and point-mutated areas. The lymph node metastasis of this tumor exhibited only the mutated p53 as well as other areas of allelic deletion, suggesting a selection for the p53 mutant in metastasis. Several investigators have reported that high-stage or invasive bladder carcinomas exhibit p53 mutations ([44] and references therein). Elegant transfection experiments reported by Thompson et al. [68] have confirmed the correlation of p53 alterations and metastatic potential. Urogenital sinus tissue from a p53-knockout mouse was transduced with retroviral vectors containing the *ras* and *myc* oncogenes. Prostatic carcinoma was observed in 100% of the p53-knockout homozygous mice and 95% of the heterozygotes, with metastatic deposits being detected in 95% of the mice. In contrast, transductions of p53 wild-type cells resulted in hyperplasia, occasional carcinoma, and no metastasis.

The role of *nm23* in urologic tumor metastasis has been subject to debate. The *nm23* gene was initially identified by its reduced expression in highly metastatic murine melanoma cell lines [62]. Low *nm23* RNA or protein expression has been correlated with a poor patient prognosis and/or histopathologic evidence of high metastatic potential in cohorts of breast, hepatocellular, ovarian, cervical, and gastric carcinomas and melanoma. In prostate carcinoma, conflicting results have been reported in cohort studies, and the relevance of this gene to metastatic progression requires clarification (reviewed in [15]). Transfection data indicating a quantitative reduction in metastatic potential have been reported in melanoma and breast-carcinoma cell lines [34, 36, 37, 52].

The Isaacs laboratory has used chromosome transfer and cloning techniques to identify a metastasis-suppressor gene for prostatic carcinoma cells. Microcell-mediated transfer of human chromosome 11 or 17 was reported to inhibit the metastatic potential of rat prostatic carcinoma cell lines [56], the former of which was subsequently localized to 11p [28]. On the basis of the identification of cDNAs in the candidate chromosomal region, the team recently reported the identification of the KAI1 gene in 11p11.2 as having functional metastasis-suppressive activity upon transfection into rat AT6.1 prostatic carcinoma cells [18]. The KAI1 gene appears to encode a cell-surface protein. KAI1 transfectants exhibited reduced invasive capacity in vitro, thus confirming that this gene has a functional rather than an immunogenic effect.

Conclusions

Translational opportunities directed at the metastatic process of urologic tumors may emanate from several different lines of investigation. Among these are the identification of genes that regulate metastatic behavior and theoretically control of all or a significant part of a cascade of events. Many of the recently identified metastasis-regulatory genes are novel, with their functions remaining unexplained to date. These genes reinforce the notion that our concept of adhesion-protease-motility factors, which may seem exhaustively defined in the literature at present, may represent only the beginning of our understanding of the metastatic process. These genes may provide new and translationally important insights. It would not be unexpected that many of the metastasis-regulatory genes identified will have impact on only a few cancer-cell types.

Another area of intense translational relevance is the process of angiogensis, as it contributes to the outgrowth of occult micrometastases that have potentially established by the time of cancer diagnosis and therapy. The redundancy of angiogenic factors represents a major obstacle to translational development. However, it can be hypothesized that many of these factors may signal the endothelial cell to proliferate and move, by common transduction pathways, beyond the cell-surface receptor. Once identified, this pathway(s) may represent an ideal target for therapeutic interception so as to interrupt the angiogenic effect of multiple stimuli.

Similarly, as colonization is involved in the outgrowth of micrometastases, the signal-transduction processes that change a traditionally inhibitory cytokine such as TGF-β to a stimulatory one may also represent a valuable translational target. This concept awaits confirmation in urologic tumors but has a strong research basis in melanoma and breast carcinoma.

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