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Analyzing the Metastatic Phenotype

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Abstract The dissemination of cells from a primary tumor, resulting in the progressive growth of metastatic carcinoma in distant sites, is the most common cause of death of cancer patients. The observations from clinical studies and the results of experimental studies using rodent tumors and human cancer cells implanted into immunodeficient host animals suggest that metastasis is not a random event, but rather the result of a sequence of selective events, many of which involve interactions with elements of the microenvironment of the primary and metastatic tumors. Analysis of the metastatic potential of a human tumor cell population has been greatly improved by the introduction of orthotopic models of tumor growth and metastasis, which have demonstrated that implanting human tumor cells into the appropriate tissue in an immunodeficient rodent can increase both tumor take and incidence of metastasis. These will be the models that should be used to validate the identity of candidate metastasis-associated genes, and to determine the value of new forms of therapy, either genetic or pharmacological, for controlling metastatic cancer growth.

Key words: metastatic carcinoma, orthotopic model, immunodeficient host, pharmacological therapy, genetic therapy

The classical definition of metastasis is the transfer of disease from a primary focus to distant sites, through the blood or lymphatics. The invasion of neighboring tissues and movement of cells around the body are not, however, unique to cancer cells; the initial transforming stimulus, whether loss of tumor suppressor gene function or activation of dominant oncogenes [Fearon and Vogelstein, 1990], does not initiate a completely new set of behavior patterns not otherwise seen somewhere, or at some time in the normal organism. For example, properties comparable to the "invasion and metastasis" of aggressive malignant cells are essential for the normal function of lymphocytes and macrophages, and in embryonic development the neural crest cells travel to various sites within the embryo to subsequently develop into distinct tissues, including peripheral nerves, Schwann cells, and melanocytes, with the movements directed by interactions with the extracellular matrix [Erikson et al., 1980]. Describing the movement of normal and embryonic and adult cells as migration and that of cancer cells as metastasis emphasizes the differences in the end results. Migration is the ordered traffic of cells from one site to another, regulated by precise intercellular communication. In contrast, metastasis can be viewed as the disorderly movement of cells from a focus of proliferating, neoplastic cells, and in this respect metastatic cells may be thought to have circumvented the regulatory controls or homeostatic mechanisms that maintain order in normal societies of tissues of multicellular organisms.

Taking breast cancer, which is one of the most common cancers in women, as an example, surgery alone will provide a cure if the disease is confined to the breast, and if the procedure removes all of the malignant cells. At the time of detection of the primary cancer, the information of greatest value for making decisions about subsequent clinical management is whether or not tumor cells are found in the regional lymph nodes. However, of the breast cancer patients classified as node-negative, up to one third will subsequently develop metastatic disease, a clear indication that micrometastases or undetected lesions were already established before treatment or removal of the primary tumor. A considerable number of tumor properties have been described as potential prognostic factors or markers that might identify the breast cancer pa-

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tients with the highest risk of developing metastases. These are generally properties or measurements made of the primary tumor; histological grading and the hormone receptor status are two routinely used markers that have proven prognostic significance, as is the presence of tumor cells in the axillary lymph nodes [Sigurdsson et al., 1990]. Other tumor markers include the degree of blood vessel density (a reflection of the angiogenic potential of the tumor), the expression of growth factor receptors, proteolytic enzyme expression, and the status of tumor-suppressor genes such as p53 and nm23 [Osborne, 1992; Gasparini et al., 1994]. How the expression of an individual marker or phenotype influences the behavior of metastatic breast cancer cells, and the value of many of these individual markers as prognostic indicators for the node-negative breast cancer patients remain to be proven by experimental studies and clinical trials, respectively. Faced with such an extensive collection of tumor properties associated with malignant progression, two general principles seem to be clearly illustrated—that breast cancer is a heterogeneous disease, and that metastasis is a process that requires expression of a variety of distinctly different genes [Weiss, 1985; Fidler, 1990].

A third important principle, that metastasis is not a random event, has its basis in both clinical observations and experimental studies. Clinical records show that certain cancers have distinct patterns of metastasis to different organs, and that these patterns are not always related to the vascular anatomy or rates of blood flow to these organs [Willis, 1973]. The predictability of the organ distribution of the metastases of breast cancer or lung cancer, for example, indicates that the development of the secondary tumors is a function of specific interactions between the disseminating cells and the sites of metastasis, rather than nonspecific, or random, growth of cells wherever they arrest. The examination of more than 700 autopsy records of breast cancer patients led Stephen Paget to propose the now venerable "seed and soil" hypothesis to explain the apparent disposition of some organs, such as the liver and lung, to develop metastases, whilst breast cancer metastases to the spleen or kidneys were rarely found. Paget's paper states, "If we can trace any sort of rule or sequence in the distribution of cancer . . . then the remote organs cannot be altogether passive" [Paget, 1889].

Injecting tumor cells labeled with a fluorescent or radioactive marker can demonstrate that, within minutes of releasing cells into the circulation, they can be detected in every organ of the experimental animal, yet the metastases will only grow in certain organs [Fujimaki et al., 1993]. The isolation of growth modulating factors from normal tissue, which can differentially stimulate the growth of rodent tumor cells metastatic to these organs, and the demonstration of specific cell binding to endothelial cells or subendothelial matrix isolated from target organs, are examples of experimental proof of the seed and soil hypothesis, and of how metastatic growth can be influenced by the tissue or host microenvironment [Nicolson, 1993]. Less well understood is whether tissue or organ derived factors play a role in the dormancy of metastases. What is the explanation for the development of metastases many years after a patient has been described as disease-free? What has kept the micrometastasis or focus of metastatic cells viable yet with no net increase in cell number, and what releases the tumor cells from the state of dormancy? Are there cytokines or growth-modulating factors present in normal organs (in addition to, or instead of, endocrine factors, Wijsman et al. [1991]), which can induce the state of dormancy in metastatic cells?

MODELS FOR THE EXPERIMENTAL ANALYSIS OF THE METASTATIC PHENOTYPE

Metastasis can be considered as the most difficult tumor cell phenotype to simulate and thereby study with in vitro techniques. Tumor growth modulation by growth factors, cytokines, chemical agents, or by cells of the immune system is routinely assessed in tissue culture, with the cells growing in monolayers, or in a semisolid medium. Some tissue culture traits have been identified as potential indicators of the metastatic phenotype; for example, invasion through a basement membrane [Albini et al., 1987] or growth in semisolid agarose. A cell's ability to grow in a semisolid medium, termed anchorage independence, is a characteristic that generally distinguishes transformed and tumorigenic cells from nontumorigenic cells. A good correlation was found between the colony forming efficiency of mouse tumor cells, or cells transformed with activated c-Ha-*ras,* in agarose cultures and their metastatic colonizing potential [Fidler et al., 1991]. For these cells, and also several human tumor cell lines, the ability to

grow in more concentrated agarose gels (0.6 to 1.2% agarose) was a more discriminating assay for identifying those cells' higher metastatic properties when assayed in the appropriate animal model [Li et al., 1989]. Several aspects of the tumor cell phenotype may explain the correlation between growth in agarose and metastatic potential, including the expression of endogenous cell-surface lectins, which could bind polymerized galactose molecules in the agar or agarose, and also the autocrine expression of growth factors [Fidler et al., 1991]. However, while in vitro assays have great practical value (in terms of saving time and money), the limitations of such experiments should always be considered; an in vitro assay generally can only evaluate a tumor cell's ability to perform one step in the multistage process of metastasis. Thus, animal models using transplantable tumors that produce a predictable number of metastases in recipient animals are the standard systems used for analyzing the metastatic phenotype, and for evaluating antimetastatic agents.

Analyses of human tumors requires the use of immunodeficient host animals, most commonly the athymic or nude mouse. Studies from this, and other laboratories have demonstrated an important principle: that implanting the human tumor tissue, as isolated cells or fragments of tumor tissue from surgical specimens, into the appropriate organ sites (termed as orthotopic implantation) will produce a higher frequency of tumor take than injection into a nonspecific or "ectopic" tissue site, usually subcutaneous injection [Fidler, 1986]. For human breast cancer cells, injection into the mammary fatpad of nude mice resulted in tumor growth at a lower inoculum and with a shorter latent interval compared with s.c. injection [Price and Zhang, 1990]. Combining the tumor cells with extracellular matrix extract known as Matrigel will also increase the tumor take for breast cancer, and a variety of other tumor types [Fridman et al., 1991; Mehta et al., 1993]. The low tumor take rate for fresh tumor tissues implanted into immunodeficient animals has been considered a serious limitation. The prospect of individualizing treatment based on the results of experimental therapies tested in nude mice against a patient's own tumor cells would seem to be untenable because of this low take rate. In one study where 262 infiltrating ductal carcinomas of the breast were implanted into nude mice, only 16 (6.1%) grew and were serially transplantable. However, 50% of these transplantable tumors expressed amplified c-*erb*B2/*neu*, an indicator of poor patient prognosis [Giovanella et al., 1991]. So that while the small proportion of breast cancers that would grow in the nude mice were not representative of the disease in general, this result does suggest that those which can be successfully transplanted may be some of the most aggressive examples. When Jessup et al. [1989] transplanted a series of colon carcinoma surgical specimens into nude mice (with a 59% take rate), the progressive growth and formation of experimental liver metastases in nude mice was correlated with the malignant progression in the patients. Using the orthotopic implantation models described in recent years has broadened the scope and potential of the immunodeficient model system and applying these techniques to rodents with more severe immuno-deficiencies, such as SCID mice and bg-nu-xid (NIH-3) [Mule et al., 1991; Garofalo et al., 1993], will further increase the value of these models for the in vivo analysis of human tumor behavior.

Of greater importance, however, the growth of the tumor in an orthotopic site can lead to a higher incidence of metastasis than is seen in mice with ectopic tumors. A high proportion (80 to 100%) of nude mice with mammary fatpad tumors of the MDA-MB-435 human breast cancer line developed metastases in lymph nodes and lungs, while fewer mice with s.c tumors (20 to 50%) presented with metastases [Price et al., 1990]. The higher incidence of metastasis from tumors growing in an orthotopic site has been shown for a variety of established tumor cell lines and explants of surgical specimens of various types of cancer, including colon, bladder, prostate, pancreatic, and renal cell carcinomas and melanoma (Table I). From the practical viewpoint, this approach has great significance, by generating reliable and relevant models of human metastatic disease, which will be of value in the development of new or improved chemotherapeutics, for example. From the philosophical viewpoint, however, the orthotopic models reinforce the concept of tumor growth, and metastasis in particular, as a consequence of interactions between the neoplastic cell and normal cells or components of the microenvironment.

MICROENVIRONMENTAL FACTORS AFFECTING METASTATIC PROGRESSION

In recent years, the theory of clonal dominance, or overgrowth of tumor populations by

TABLE I. Examples of Models for Human Cancer Metastasis Using Orthotopic Implantation in Nude Mice

Cancer	Sites of metastasis	Reference
Bladder	Lymph nodes, lungs	Aherling et al., 1987 Fu et al., 1991
Breast	Lymph nodes, lungs, brain, adrenal, muscle	Price et al., 1990
Colon	Liver, lymph nodes	Morikawa et al., 1988
Lung	Lung, lymph nodes	Wang et al., 1992
Melanoma	Lymph nodes, lungs, brain	Cornil et al., 1989
Pancreatic	Liver, lung, lymph nodes	Vezeridis et al., 1989 Fu et al., 1992
Prostate	Lymph nodes	Stephenson et al., 1992
Renal cell	Lymph nodes, lungs	Naito et al., 1987
Stomach	Liver, lung	Furukawa et al., 1993

metastatic subpopulations, has been developed, based on experiments using the techniques of genetic marking (transfection with drug-resistance genes) or selection for drug-resistant variants [Miller et al., 1987; Kerbel, 1990]. Metastatic cells generally have a growth advantage over non- or weakly metastatic cells within the primary tumors, and eventually can become the dominant population. The study by Staroselsky et al. [1992], using the technique of genetic tagging by introduction of a neomycin resistance gene, showed that distinct clones of cells were the predominant populations in the tumors recovered from different organs of nude mice injected with a human renal cell cancer, with the same pattern of clonality found in the subrenal capsule (orthotopic) tumors and resulting metastases. In contrast, there was less concordance the patterns of clonality in local tumors and metastases resulting from s.c. (ectopic) injections. This demonstrates, therefore, that the tumors growing in different sites in nude mice can consist of different subpopulations, selected presumably by their ability to proliferate in the particular environment. One explanation for clonal selection in heterogenous tumors is that in the course of malignant progression, the responsiveness of the tumor cells to cytokines is altered, by increased responsiveness to stimulating factors and/or decreased responsiveness to inhibitory factors such as TGF-β, that are present in the tissue environment [Kerbel, 1990]. Experimental evidence is provided in analyses of growth factor and cytokine responses of cell lines representing different stages of melanoma progression, studies which have suggested that fibroblast-derived factors may be involved. One candidate factor is IL-6, since this cytokine was shown to inhibit the growth of early stage melanoma cells, yet stimulated the growth, and is an autocrine growth factor for late stage and metastatic melanoma [Lu and Kerbel, 1993].

Tumors grown in the subcutis of nude mice commonly develop an extensive fibrous capsule, while those in the orthotopic site have a less extensive or no fibrous capsule [Morikawa et al., 1988; Naito et al., 1987]. Whilst a study of the integrity of basement membranes surrounding tumors growing in different organ sites did not indicate that the fibrous capsule around s.c. tumors presented a physical barrier to tumor cell invasion [Sekikawa et al., 1988], the stromal matrix and stromal fibroblasts have been shown to modulate the expression of proteolytic enzymes by the tumors. The invasive phenotype of colon cancer and renal cell cancer cells selected for metastatic potential in nude mice (following orthotopic implantation) was found to be modulated by fibroblasts from different organs. Coculture with skin-derived fibroblasts reduced the level of collagenase type IV (gelatinase) produced by the tumor cells in vitro, while growth with fibroblasts from the appropriate normal organ did not reduce the production of latent and active forms of the 64 kDa type gelatinase; for both of these tumor lines metastases were not found, or were less frequent following s.c. injection in nude mice, implying that factor present in the s.c. environment may have reduced the invasive and metastatic potential of the cells. Two fibroblast derived cytokines, IFN-β and TGF-β have been implicated in the regulation, either by inhibition or stimulation, respectively, of the gelatinase activity of the tumor cells in vitro [Fabra et al., 1992; Gohji et al., 1994]. These are two examples of cytokines with multiple effects on tumor cells, at least in vitro, and that potentially can influence growth (direct growth modulation or via autocrine factors), neovascularization of the primary or metastatic tumor (paracrine release of angiogenic factors),

and the activity of proteolytic enzymes important in the invasive components of the metastatic cascade [Liotta and Stetler-Stevenson, 1990]. The use of *appropriate* animal models, and also techniques such as in situ hybridization to localize expression of specific genes, will elucidate the role of stromal cell-derived factors in malignant progression.

IDENTIFICATION OF METASTASIS-ASSOCIATED GENES

In recent years different approaches have been taken in the search for genes which are involved in metastasis. Differential hybridization techniques have identified a number of candidate genes, some expressed preferentially or in higher amounts in the metastatic cells, or conversely, some that are down-regulated in metastatic cells, i.e., putative suppressor genes [Hart and Easty, 1991; Pencil et al., 1993]. The most widely studied candidate "metastasis suppressor" is the nm23 gene, initially identified in the K1735 mouse melanoma system [Steeg et al., 1988]. While another study using the K1735 melanoma and other metastatic tumors was unable to corroborate the correlation between low nm23 expression and metastatic potential [Radinsky et al., 1992], a low nm23 expression level has been associated with poor patient prognosis in a number of clinical trials, most notably of breast cancer [Hennessy et al., 1991]. Introducing nm23 into the metastatic MDA-MB-435 cell line (which has low endogenous expression) reduced the capability to metastasize in nude mice. The evolutionary conservation of nm23, indicated by its homology with a nucleoside diphosphate kinase of *Dictyostelium,* would suggest that it has a fundamental role in cell growth and regulation [Steeg et al., 1993], yet how that may be associated with metastasis in some tumor cells, e.g., breast, and not others, e.g., colon [Haut et al., 1991] (is this a consequence of tissue-specific regulation?), remains to be determined.

Differential display has been successfully used to identify genes associated with the malignant progression of breast cancer, and two candidate suppressor genes have recently been identified. The integrin α6 subunit was found to be expressed at reduced levels in breast cancer cell lines compared with normal mammary epithelial cells [Sager et al., 1993]. This integrin subunit is expressed at variable levels in breast cancer biopsies, with alterations coinciding with the loss of basement membrane [Natali et al., 1992]. While not identifying this particular integrin as critical in mediating cell-matrix interactions, Petersen et al. [1992] demonstrated that in vitro normal mammary epithelial cells, but not breast cancer cells, will respond to basement membrane proteins with growth arrest and expression of markers of differentiation (including formation of basement membrane). In addition to functioning as adhesion receptors, the key role of integrins in signaling information from the microenvironment that can modulate normal and malignant cell growth and differentiation is becoming increasingly apparent [Damsky and Werb, 1992]. A second candidate tumor suppressor gene identified by Sager and colleagues [1993; Zou et al., 1994] is a novel member of the serpin family of protease inhibitor, termed maspin. When transfected into metastatic MDA-MB-435 breast cancer cells, maspin was found to reduce the invasive and metastatic potential [Zou et al., 1994]. These two examples cited here were chosen since the genes detected by differential display both potentially influence the tumor cells' interactions with the environment, by adhesion and/or signalling though an integrin subunit and expression of a protease inhibitor.

PROSPECTS

Differential display will identify a host of genes, some known and some novel, that are associated with the metastatic phenotype. Since there is a wealth of literature to support the hypothesis that metastasis is influenced by interactions between the tumor cells and the microenvironment, the genes of most interest will probably be those that can be shown to have an essential function at some stage of these interactions. Confirmation of the role of any candidate metastasis gene will depend on the assay system (or systems) chosen—the results will probably only be as good as the model used, and the most relevant information will come from the most relevant and reliable metastasis models. These will be the models to use in testing whether, for example, re-expression of a candidate metastasis-suppressor gene, or down regulation of a metastasis promoting gene with antisense constructs can reduce or totally inhibit the metastatic properties of a tumor growing in the appropriate tissue environment. The value of these studies will be in confirming the role of a particular gene as a prognostic indicator (and therefore its value in deciding which node-negative breast cancer

patients, for example, would benefit most from intensive therapy) as well as confirming the possibility of genetic regulation (pharmacologically or by gene therapy) for controlling the growth and dissemination of metastatic cells.

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