REVIEW

Metastasis Suppressor Genes

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Within the heterogeneous cell population of malignant neoplasms are cells with the ability to invade and metastasize. Metastatic propensity is distinctly separate from tumorigenicity alone. The complexity of the metastatic process suggests that it is controlled at the genetic level via the activation and/or deactivation of multiple genes. It is now generally accepted that there are loci in normal cells that can suppress the tumorigenic phenotype and that can be inactivated by mutation. Recent evidence from somatic cell hybridization studies and DNA transfection experiments as well as the isolation of complementary DNA clones by subtractive hybridization and by differential screening predicts that an analogous (but distinct) set of metastasis suppressor genes may exist within tumor cells that can inhibit invasion and metastasis. The interaction of the gene products of potential stimulatory and inhibitory metastasis genes may be critical in determining the metastatic phenotype of tumor cells. [J Natl Cancer Inst 82:267–276, 1990]

Metastasis is a complex process in which tumor cells colonize sites distant from the primary tumor. Tumor metastasis remains the major cause of morbidity and death for patients with cancer (*1*). The mechanisms responsible for the metastatic behavior of tumor cells are not fully understood; their elucidation will hopefully lead to improved methods to diagnose and treat metastatic disease. Several general principles have guided the investigational approaches to tumor cell invasion and metastasis. The general phenomenon of tumor cell heterogeneity has been recognized for some time (*2–4*). In particular, it has been noted that not all tumor cells are capable of metastasis and that metastatic potential varies among particular cells of a tumor. Thus, although cancer cells must be tumorigenic to grow as a metastatic colony, the metastatic phenotype is independent from the tumorigenic phenotype.

A second general principle is that a metastatic colony is the end result of a complex series of tumor–host interactions that follow primary tumor initiation and progression. The metastatic tumor cell must evade host defenses while it detaches from the primary tumor, invades the primary tumor border and adjacent host tissue barriers, intravasates the vascular wall or lymphatic channel, survives the mechanical stress of the circulatory system, extravasates the vascular wall, enters an organ parenchyma, and colonizes at a distant site. Both genetic and epigenetic events have been implicated in the process by which individual cells acquire the characteristics for invasion, dissemination, survival, and growth at the metastatic site (*5,6*), and it has been proposed that both mechanisms might operate in concert (*7,8*). If, indeed, specific genetic changes occur in a tumor cell that are at least in part responsible for the metastatic phenotype, these changes should be stable enough to be reflected in the cell's progeny. Given the complex nature of the metastatic process, it is likely that the process involves multiple gene products. A major emphasis has been placed on the identification of so-called "metastasis genes" or "metastogenes" (*9*) that elicit or augment the metastatic phenotype (*10*). Recently, more attention has been given to the possibility that genetic control of metastasis is also exerted via the deactivation of specific genes.

In this review, evidence for the role of tumor suppressor genes in malignancy will first be presented, followed by a consideration of the potential biochemical mechanisms involved in tumor invasion and metastasis and the gene products that play a role in the induction and maintenance of the metastatic phenotype. Finally, current approaches to identify genes that may inhibit or suppress metastasis will be discussed.

Tumor Suppressor Genes

Klein (*11*) and Hansen and Cavenee (*12*) recently reviewed in detail evidence supporting the viewpoints that cancer is a genetic disorder and that predisposition to cancer is the result in some cases of the inheritance of recessive mutant alleles at loci for phenotypic suppressors of tumorigenesis. Klein (*11*) proposed that tumor suppressor genes may act at either the transcriptional or the posttranscriptional levels and summarized the evidence for tumor suppressor genes in a variety of systems as follows: (*a*) In general, it has been found that the fusion of normal and malignant cells leads to the suppression of the tumorigenic phenotype and that reappearance of tumorigenicity is accompanied by specific chromosome losses. (*b*) Nontumorigenic revertants of virally and chemically induced transformants have been isolated that do not appear to be generated by the loss of the transforming gene or by

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its down-regulation (i.e., decreased expression). (*c*) Differentiation blocks can be bypassed by the temporary down-regulation of temperature-sensitive oncogenes or by exposure to differentiation-inducing signals for a sufficient period of time. (*d*) Regulatory sequences capable of modulating oncogene expression have also been identified. (*e*) Diffusible products of normal cells appear to be able to inhibit tumor growth. (*f*) Certain forms of cancer tend to cluster in families, with the genetic predisposition to cancer often behaving as an autosomal dominant trait; in particular, the loss or mutational inactivation of "recessive cancer genes" appears to play an essential role in the genesis of a variety of childhood tumors such as retinoblastoma.

Retinoblastoma has been used as a prototype biological model for the study of human cancers that are genetically inherited with an autosomal dominant phenotype, involving tumor suppressor genes that are recessive at the cellular level. In 1973, Comings (*13*) proposed a general theory of carcinogenesis, of which the critical features were that (*a*) all cells possess multiple structural genes capable of coding for transforming factors that can release the cell from its normal constraints on growth, and (*b*) in adult cells, these genes are suppressed by diploid pairs of regulatory genes. Comings further proposed that the transforming factors are temporarily activated at some stage of embryogenesis and that tumors arise as the result of a double mutation of any set of regulatory genes releasing the suppression of the transforming factor, leading to the transformation of the cell. The theory was based in part on the observations and hypotheses of Knudson (*14*), who studied the rate at which two types of retinoblastoma, familial and sporadic, appeared in young children. It was proposed at that time, and has since been confirmed by recent molecular analysis, that all cases of retinoblastoma are caused by two mutational events. The mutations have been mapped to a single locus, RB1, on human chromosome 13 band q14 (*15,16*). Retinoblastomas develop only when the function of both alleles is destroyed. Patients with the familial form of retinoblastoma carry one germline mutation, such that all cells have one normal allele and one mutant allele. In these patients, the germline mutation is a predisposing mutation; the tumor develops when the normal allele of a retinal cell is lost or acquires a mutation. Most patients with familial retinoblastoma develop bilateral retinal tumors because mutations arise in more than one retinal cell in both eyes. In the noninherited sporadic form of retinoblastoma, a single retinal cell acquires somatic mutations of both RB1 alleles. In these cases, since there is no predisposing germline mutation of one of the alleles, patients develop a single, unilateral tumor. In either the familial or the sporadic form of retinoblastoma, the second (somatic) mutation usually involves a chromosomal mechanism resulting in homozygosity for most of the long arm of chromosome 13 (*15*).

Children who inherit the deletion of one RB1 allele from a parent carry the risk of developing retinoblastoma only during the first few years of life. If the second allele is not lost by a somatic mutation by the age of approximately 5 years, all the retinoblasts will have terminally differentiated into retinocytes that cannot divide. Nonetheless, those individuals carrying the RB1 germline mutation have a high incidence of nonocular tumors, often osteosarcoma. It was recently demonstrated that osteosarcoma is also associated with the double loss of the RB1 gene (*17*). Abnormalities in the structure and expression of the RB1 gene have also been identified in small cell cancer of the lung (*18*) and in human breast cancers (*19*). This finding suggests that the RB1 gene is a pleiotropic suppressor gene, required for the normal maturation of several cell types (*11*).

A human complementary DNA (cDNA) gene probe has been isolated with properties consistent with those of the RB1 gene (*17,20,21*). Sequence analysis of the cDNA clones demonstrated a long open reading frame encoding a hypothetical protein with features suggestive of a nuclear phosphoprotein associated with DNA binding activity (*22*). It was subsequently demonstrated that the retinoblastoma gene product forms a specific complex with the simian virus 40 large T antigen (*23*) as well as with E1a, the transforming protein of adenovirus (*24*). A point mutation in the RB1 gene has been identified that results in the elimination of 35 amino acids from the gene product; the latter cannot complex with E1a (*25*). The interactions of the retinoblastoma gene product with large T antigen and with E1a are the first examples of a direct physical link between oncogenes and a tumor suppressor gene. Other potential tumor suppressor genes, such as p53, may also complex with nuclear oncoproteins (*26*). Such interactions have recently been reviewed, and it has been noted that diverse nuclear oncoproteins have limited regions of amino acid sequence homology that may be sites of binding to tumor suppressors (*26*). A potential suppressor gene has also been reported that provides resistance specific to activated ras genes (*27*). A human cDNA was recovered from a revertant of Kirsten sarcoma virus-transformed NIH 3T3 cells. This cDNA encodes a protein with structural similarities to ras proteins. Thus, such a hypothetical protein may compete with ras proteins for a common target or regulatory protein. The oncogene products may thus inactivate specific cellular proteins that control cellular proliferation.

It was recently demonstrated that the neoplastic phenotype of retinoblastoma cells and osteosarcoma cells, both of which contain inactivated endogenous RB1 genes, can be suppressed by the transfection of the normal retinoblastoma cDNA (*28*). Tumorigenicity, as well as soft agar colony formation, was altered by the expression of the RB1 gene, providing evidence for an essential role of the RB1 gene in the suppression of tumorigenesis.

Other loci that can suppress the tumor phenotype and that are inactivated by mutation have been postulated for other tumors. A gene localized at human chromosome 11 band p13 appears to play a role in Wilms' tumor (*28*); a normal human chromosome 11 introduced into a Wilms' tumor cell line by the microcell transfer technique suppressed the ability of the cells to form tumors in nude mice (*29*). Also localized to chromosome 11 has been the gene for multiple endocrine neoplasia type 1, a predisposition to hyperplasia of the parathyroid glands and to hyperplasia and tumors of the anterior pituitary and the pancreas that is inherited as an autosomal dominant trait (*30*). Reduction to homozygosity of genes on chromosome 11 has been correlated with human breast neoplasia, including a significant association with tumors that lack estrogen and progesterone receptors and that present with distal metastasis (*31*). Deletions of sequences from the short arm of chromosome 11 have been detected in several other neoplasms of epithelial origin [reviewed in (*31*)]. Other examples of potential tumor suppressor genes of the "recessive" type include a locus for human acoustic neuroma on chromosome 22

(*32*) and a locus for familial adenomatous polyposis and a locus for sporadic colorectal adenocarcinomas on chromosome 5 (*33,34*). The development of colorectal carcinomas has also been associated with allelic loss on chromosomes 17 and 18 (*35–37*). Thus, several genes may be involved in the suppression of colon tumors (*36–38*).

A recessive cancer suppressor gene has also been postulated to control the activity of an inhibitor of angiogenesis (*39*). The direct role of this gene and the other (non-RB1) proposed tumor suppressor genes in inhibiting tumorigenesis has not been demonstrated and must await the molecular isolation of the specific genes and the appropriate DNA transfection studies.

Gene Products Involved in Tumor Invasion and Metastasis

Metastatic propensity is distinctly separate from tumorigenicity. It can be hypothesized that genes that inhibit metastasis, so-called "metastasis suppressor genes," are distinct from tumor suppressor genes. Genes that can influence malignant properties, such as invasiveness, metastatic propensity, and the ability to generate an immune response, have been classified as "modulator genes" by Klein (*11*). Such modulator genes should include both "metastasis genes," which elicit or augment the metastatic phenotype, and "metastasis suppressor genes," which can inhibit metastasis. A better understanding of the metastasis suppressor genes and the potential sites of action for their gene products will hopefully be achieved once the underlying mechanisms responsible for the induction and maintenance of metastatic behavior are fully elucidated.

Considerable progress has been made in recent years to define the biochemical mechanisms of tumor invasion and metastasis (*7,10,40*). An underlying principle is that cancer invasion and metastasis represent a complex, multistep process. Following local invasion of adjacent host tissue barriers, a metastatic tumor cell must invade the vascular wall or lymphatic channels to disseminate. After intravasation, tumor cells in the circulation must be able to evade host defenses, survive the mechanical trauma of the blood flow, and arrest in the venous or capillary bed of the target organ, after which the tumor cell must again invade the vascular wall to enter the organ parenchyma. The extravasated tumor cell must then grow in the new site, which may have an environment different from that of the tissue of origin (*10,41,42*). To successfully traverse all the steps of this process and to initiate a metastatic colony, the tumor cell must express the right combination of gene products. Given the myriad possibilities, it seems unlikely that there is a unique gene product that is universally essential to the metastatic process; rather, it would appear that several gene products, differing from one metastatic cell to another, but having certain properties in common, must be expressed.

Much attention has been focused on the interaction of the metastatic tumor cell with the extracellular matrix and, in particular, with the basement membrane through which it must traverse. A three-step hypothesis that describes the sequence of biochemical events during tumor cell invasion of the extracellular matrix has been proposed (*43*). The first step is tumor cell attachment via cell-surface receptors that specifically bind to components of the matrix, such as laminin (for the basement membrane) and fibronectin (for the stroma). In the second step, hydrolytic enzymes locally degrade the matrix, including the attachment components. This degradation may variably be achieved by the anchored tumor cell's secretion of hydrolytic enzymes, by induction of host cells to secrete enzymes, or by the activation of proenzymes already present in the matrix. The third step is tumor cell locomotion into the region of the matrix modified by proteolysis. The cyclic repetition of these three steps is probably required during continued invasion through the matrix and during the rest of the metastatic process.

As an outgrowth of this hypothesis, several gene products that are augmented in metastatic cells have been identified. Laminin receptors are altered in number or degree of occupancy in a variety of human carcinomas (*10,44,45*); while the laminin receptors of normal epithelium are polarized at the basal surface and occupied with laminin in the basement membrane, the laminin receptors on invading carcinoma cells are amplified and are distributed over the entire surface of the cell (*10,44*). The steady-state levels of laminin receptor messenger RNA (mRNA) are increased in metastatic breast and colon carcinoma cells (*46,47*). The laminin receptor has been shown experimentally to play a role in hematogenous metastasis (*48*); treatment of tumor cells with the receptor-binding fragment of laminin inhibited lung metastases.

Other cell-surface receptors have been implicated in the adhesion process. Treatment of melanoma cells ex vivo with the peptide sequence GRGDS (glycine–arginine–glycine–aspartic acid–serine), which is present in fibronectin as well as other adhesion-promoting proteins, inhibited experimental metastasis (*49*). Pretreatment of tumor cells with a heparin-binding fragment of fibronectin has also been shown to inhibit experimental metastases (*50*). These results suggest a role for the integrin family of cell-surface glycoproteins (*51,52*) as well as for a cell-surface proteoglycan (*50*) in the metastatic process, although these cell-surface receptors have not yet been demonstrated to be augmented in metastatic cells.

Invasive tumor cells secrete matrix-degrading proteinases. Two types of enzyme have been identified that specifically degrade basement membrane components. One is an endoglycosidase that is specific for heparan sulfate; its activity has been correlated with metastatic potential (*53,54*). The other type of enzyme is collagenolytic. Basement membrane-specific type IV collagen is not susceptible to degradation by interstitial collagenases, but it is specifically degraded by type IV collagenase. The level of type IV collagenase is augmented in many highly metastatic tumor cells (*55*). The expression of type IV collagenase correlated with the metastatic potential of murine tumor cell hybrids (*56*) and oncogene-transfected cells (*57*). Laminin was shown to increase the release of type IV collagenase from malignant human melanoma cells (*58*), suggesting that tumor cell binding to laminin, which is an early step in basement membrane invasion, can induce a later step, i.e., the collagenolytic dissolution of the basement membrane.

The role of collagenases and inhibitors of collagenases in tumor cell invasion has been reviewed (*59*). The unrestrained activity of collagenases may be involved in tumor cell invasion. Since these enzymes are secreted as zymogens, their activity may be modulated by factors affecting their conversion from latent to active form. Metalloproteinase-mediated collagenolysis may also

be regulated through naturally occurring inhibitor proteins, such as the tissue inhibitor of metalloproteinase (TIMP) (*60*). TIMP is a ubiquitous glycoprotein with an approximate molecular weight of 29,000; it forms a 1:1 stoichiometry with activated interstitial collagenase. Furthermore, TIMP can be synthesized by the same cells that secrete interstitial collagenase (*61,62*). As a consequence, net collagenolytic activity results when activated collagenase levels exceed TIMP production. Increased activity of interstitial collagenases associated with malignancy has been reported (*59,63,64*). In addition, levels of mRNA for the protease transin (also known as stromelysin) were reported to be increased in malignant tumors (*65*). TIMP can inhibit stromelysin as well as interstitial collagenase (*66,67*). Consistent with the above findings, an inverse correlation between TIMP levels and the invasive potential of murine and human tumor cells has been reported (*68,69*). TIMP inhibited the invasion of murine melanoma cells in an in vitro amnion invasion assay system, and the injection of TIMP into mice significantly inhibited metastatic lung colonization by the melanoma cells in vivo (*70*). Furthermore, it was recently demonstrated that the decrease of TIMP mRNA levels in nontumorigenic and noninvasive Swiss 3T3 cells via anti-sense RNA resulted in conversion to tumorigenic cells with metastatic potential (*71*). The increased content of another metalloproteinase inhibitor in desmoplasia and in human cirrhotic liver has been proposed as a possible mechanism for the resistance to metastasis of certain tissues (*72*). Recently, a metalloproteinase inhibitor, TIMP-2, has been identified that can complex with type IV collagenase and inhibit activity of the enzyme (*73,74*). Thus, the following hypothesis can be proposed: A family of metalloproteinase inhibitors may act to suppress metastasis via the inhibition of specific metalloproteinases involved in tumor cell invasion.

In addition to the enzymes that can degrade basement membrane components and collagen in the stroma, the increased activity of cathepsin B has been associated with metastasis (*75–77*). It thus appears that a cascade of tumor cell proteinases may participate in the degradation of the extracellular matrix, either by mechanisms involving increased synthesis and secretion of proteases or by decreased inhibition by anti-proteinases.

Tumor cell motility factors also appear to be associated with metastatic cells (*10,78*). An autocrine motility factor was recently shown to induce the extension of cell pseudopodia before cell translocation; the number of laminin receptor and fibronectin RGD (arginine–glycine–aspartic acid) recognition sites was markedly increased in induced pseudopodia (*79*). These data, coupled with the observation that exposure to laminin can induce type IV collagenolytic activity in melanoma cells (*58*), suggest that the various steps of the invasion process are interrelated and are regulated in a complex manner.

Tumor cell-surface lectins may also play a role in the metastatic process. A galactoside-binding lectin found in melanoma, fibrosarcoma, and angiosarcoma cells is differentially expressed in tumor cell variants that have the greatest metastatic potential (*80,81*). This lectin is a chimeric gene product with homology to an IgE-binding protein as well as to collagen (*82*) and may promote the formation of multicell emboli in the circulation. It was recently observed that Galα1-3Gal cell-surface residues, which are not normally expressed at the surface of human cells, are expressed at the surface of malignant human cancer cells (*83*). Furthermore, human natural anti-α-galactosyl antibodies inhibited cell attachment to endothelium and to laminin, suggesting that human natural anti-Gal antibodies play a role as a natural antitumor defense system (*83*).

Genetic Induction of Metastasis

It is apparent from the above discussion that the metastatic process involves multiple gene products. It has been proposed that a cascade or coordinated group of gene products expressed above a certain threshold level may be required for a tumor cell to successfully proceed through the steps in the metastatic process (*10*). A corollary of this concept is that the acquisition of the metastatic phenotype may not necessarily depend on the induction of unique, specific genes (qualitative changes), but rather may merely depend on the "turning up" (quantitative changes) of expression of certain genes (*8*).

Genetic induction of the metastatic phenotype has been observed experimentally by somatic cell fusion studies and by DNA transfection. Critical to the study of induction of metastatic behavior are the availability of suitable assays to determine if a cell line is metastatic and an assessment of whether the intrinsic aggressiveness of the tumor cells or their interaction with the host immune system is being measured. Metastasis assays have been reviewed (*7,84*); a common feature of the assays is the injection of cells into an animal host and subsequent determination of metastatic colonies. Two major considerations for these assays are the choice of animal hosts and the type of injections. In the absence of syngeneic animals, young, athymic mice are used to reduce immunologic barriers. Nonetheless, sensitivity to natural killer cells and macrophage-mediated cytotoxicity must be considered. How metastasis assays are conducted varies greatly. In so-called "spontaneous metastasis assays," cells are injected subcutaneously, and the ability of cells to form a primary tumor and subsequently spread from that site, gain access to the circulatory system, and establish distant metastatic colonies is measured. Since the primary tumor will often kill the animal, some investigators excise the primary tumor after formation, thus extending the period during which the animal can be evaluated for metastasis. Another variation of these assays is to recapitulate the natural history of the tumor by injection of the tumor cells into their tissue of origin.

In contrast to the spontaneous metastasis assays, "experimental metastasis assays" involve the intravenous injection of cells into the animal. In this case, the assays duplicate the later steps in the metastatic process. It is perhaps not surprising that results from spontaneous and experimental metastasis assays often correlate (*7*), since many of the same gene products may be involved in the various steps of tumor invasion and metastasis.

The contribution of the host immune surveillance system cannot be underestimated (*85*). Genes that regulate the major histocompatibility antigens on the tumor cell surface have been shown to affect the metastatic process. Highly metastatic clones of murine tumors express the H-2D, but not the H-2K, gene of the major histocompatibility complex (MHC) system. Modulation of the expression of the MHC genes altered the metastatic competence of tumor cells (*86*). Furthermore, transfection of the H-2K gene into the metastatic cells, with resultant expression of H-2K antigens, abrogated the metastatic properties of the tumor cells (*87,88*). The immunogenicity of H-2K-expressing cells and their

nonmetastatic properties appear to be causally related; H-2K transfectants can be potent inducers of, and susceptible targets for, antitumor cytotoxic T cells (*86*). MHC gene expression appears to be controlled by c-fos. Thus, the c-fos oncogene is differentially expressed in nonmetastatic clones that express H-2K, and the transfection of c-fos results in the transcriptional activation of H-2K mRNA (*86,89*). These studies imply that c-fos may suppress metastasis, acting through a mechanism by which the immunogenicity of the tumor cell is increased via expression of the H-2K antigen.

Other evidence indicates that tumor cells may express genes that enhance their immunogenicity with a consequent suppression of metastasis. Oncogene-transfected murine cells that were metastatic in nude mice were tumorigenic but not metastatic in immunocompetent mice; metastatic capacity in immunocompetent mice was restored on transfection of human tumor DNA into the cells (*90*). The transfected gene has not been identified; it has been proposed that it may play a role, not in the intrinsic aggressiveness of the metastatic cell, but in its interaction with the host immune defense system (*91*).

Immunologic considerations aside, presumably, a single gene could induce the complete metastatic phenotype (i.e., intrinsic aggressiveness) by one or more of several proposed mechanisms (*91*): (*a*) In the additive hypothesis, the "metastasis gene" could confer the final factor to cells that are already expressing all the other necessary gene products for metastasis; (*b*) the gene could induce a genetic instability that results in the production of metastatic variants that are selected in vivo; or (*c*) the metastasis gene could induce the expression of a cascade of cellular gene products that are sufficient for the metastatic phenotype.

The genetic induction of the metastatic phenotype has been described in cell fusion experiments. While, in general, the fusion of normal cells with neoplastic cells results in nontumorigenic hybrids [reviewed in (*11*)], the fusion of bone marrow-derived normal cells with tumor cells may give rise to metastatic behavior (*92–94*). It has been proposed that the specific properties derived from normal lymphoid cells, i.e., their tendency to circulate and to home to certain tissues as well as their invasive potential when introduced into noninvasive but tumorigenic cells, cause the latter cells to become invasive and metastatic (*93*). Collard et al. (*94*) fused nonmetastatic mouse lymphoma cells with human normal lymphocytes or with human leukemic T lymphoblasts. Both types of fusions resulted in highly invasive human–mouse T-cell hybrids that metastasized in nude mice. Continued in vitro selection for invasive cells resulted in the isolation of invasive hybrids that had lost all human chromosomes except chromosome 7, suggesting that genes involved in invasiveness and presumably sufficient to establish metastatic potential in T-cell hybrids are located on human chromosome 7. Both the epidermal growth factor (EGF) receptor and the platelet-derived growth factor (PDGF) A chain are located on this chromosome. Structural or numerical alterations of chromosome 7, often accompanied by enhanced expression of the EGF receptor or PDGF A chain, have been correlated with invasiveness and metastatic potential (*95–99*) in a variety of tumors.

With the advent of transfection techniques, the systematic induction of metastatic behavior in cells has been possible (*100*). The independence of metastatic potential from tumorigenicity has been well documented (*84*). Therefore, when the ras oncogene was identified as a gene capable of inducing tumorigenicity (*101*), it was not clear if a single gene such as ras could induce the complete metastatic phenotype as well. Nonetheless, the experimental evidence, which has been extensively reviewed (*7*), now clearly indicates that transfection by the ras oncogene is sufficient to confer metastatic potential in a variety of cell types, including diploid cells. Significantly, cells transfected with ras have been shown to secrete greater quantities of type IV collagenase (*57,100*). The enhanced metastatic potential of ras-transfected cells has also been correlated with altered growth factor sensitivity (*102,103*). While the acquisition of growth autonomy is commonly associated with the tumorigenic phenotype, specific alterations in responsiveness of metastatic cells to growth factors have been noted. Rare metastatic variants of ras-transfected CCL39 fibroblasts from the Chinese hamster lung acquired the ability to grow in serum-free conditions (*102*). The latter study suggests that ras can induce in the cells an ability to divide in an autonomous fashion, independent of growth factors. It is not clear, however, whether the induction of the metastatic phenotype in the transfectants was causally related to the growth autonomy. The induction of the metastatic phenotype may have been related to the acquisition of other cellular attributes, such as collagenase expression. In ras-transfected murine 10T½ cells, both metastatic and nonmetastatic cells showed a diminished responsiveness to basic fibroblast growth factor, PDGF, and EGF (*103*). However, while nonmetastatic transfectants exhibited the normal, inhibitory response to transforming growth factor β (TGF-β), DNA synthesis was stimulated in TGF-β-treated metastatic cells. Thus, the metastatic cells demonstrated an altered sensitivity to a specific growth factor, implying that an altered TGF-β receptor may be involved in the acquisition of the metastatic phenotype in these cells. Retinoblastoma cells are also resistant to TGF-β, presumably due to a loss of receptors for that growth factor (*104*).

Oncogenes other than ras, including mos, raf, src, fes, and fms, can induce metastatic potential upon transfection (*105*), although the metastasis-inducing effects of these oncogenes may act via a common ras-dependent pathway (*106*).

The correlation of oncogene expression with human tumor metastatic aggressiveness has recently been reviewed (*10*). Increased expression and/or amplification of oncogenes have been reported in a variety of tumor systems (*10*). Amplification of the HER-2/neu oncogene has been correlated with metastases in human breast carcinoma (*107,108*), while N-myc amplification has been associated with rapid progression of neuroblastomas (*109*). Increased expression of ras has been detected in various cancers, including breast and colon cancers (*10*). The L-myc gene is amplified in small cell carcinomas of the lung (*110*).

Restriction fragment length polymorphisms (RFLPs) of oncogenes have also been reported in metastasis. Elevated frequencies of specific alleles of the c-Ha-ras gene have been reported with a variety of cancers, including breast, lung, and colorectal cancers (*111–114*). However, these correlations are not necessarily of prognostic importance (*38*). L-myc RFLPs have also been associated with metastasis of human lung and renal cancers, but not with metastasis of colorectal cancers (*115–117*). Oncogene alterations may merely be a hallmark of the genetic instability of tumors and may not be causally related to the metastatic phenotype. However, there appears to be some specificity for different

oncogenes with distinct histologic types of tumor. Thus, if oncogenes are indeed important in human tumor metastasis, the effect of a particular oncogene may depend on the genetic background of the host cell.

Approaches To Identify Metastasis Suppressor Genes

A gene that in some way inhibits the formation of metastasis may be defined as a metastasis suppressor gene. Since tumorigenicity and metastatic propensity are independent processes, it follows that metastasis suppressor genes are distinct from tumor suppressor genes. A metastasis suppressor gene may function by increasing the immunogenicity of the tumor cell in the host, such as described above for the H-2K antigen (*86–89*). Another type of metastasis suppressor gene would be one encoding a protein that directly inhibits a gene product intrinsically involved in the metastatic process. With this definition, TIMP and TIMP-2 (*71,73,74*) may be classified as tumor suppressor genes, since they inhibit metalloproteinases that are up-regulated (i.e., expressed more) in metastatic cells. This definition is limited in that it is dependent on the identification of specific genes that are up-regulated in metastasis. Another type of metastasis suppressor gene would be one inhibiting a protein that regulates a cascade of metastatic properties or that interacts directly with the RNA or DNA of metastasis-associated genes. A combination of cell fusion, DNA transfection, and RFLP analysis may be useful in identifying the latter class of metastasis suppressor genes.

As described in the first section of this review, cell fusion studies provided early indications that tumor suppressor genes exist. With tumor suppressor genes, the tumorigenicity of the cell hybrid is abrogated. Since tumorigenicity and metastatic propensity are independent processes, the identification of metastasis suppressor genes from cell fusion studies would require that the cell hybrids be tumorigenic but nonmetastatic. Several cell fusion studies have provided indirect evidence for the existence of metastasis suppressor genes. Ramshaw et al. (*118*) demonstrated that the fusion of rat metastatic mammary carcinoma cells with various nonmetastatic cells resulted in hybrid clones in which the metastatic potential was suppressed; i.e., the metastatic phenotype behaved as a recessive characteristic. Similarly, metastatic behavior was recessive when mouse melanoma cells were fused with normal cells (*119*), when mouse metastatic lung carcinoma cells were fused with tumorigenic but nonmetastatic mouse L cells (*120*), and when mouse metastatic melanoma cells were fused with normal cells (*56*). In the latter case, type IV collagenase activity was suppressed along with the metastatic potential.

DNA transfection studies have also provided some indirect evidence for metastasis suppressor genes. Muschel et al. (*121*) found that transfection of the ras oncogene alone could induce the metastatic phenotype only in certain types of recipient cells. C127 cells, derived originally from a murine mammary tumor, could be transformed by activated ras into tumorigenic cells expressing high levels of $p21^{ras}$, but they remained nonmetastatic in either experimental or spontaneous metastasis assays. These data suggested that alterations other than those involving oncogenes in the genetic background of the cell are important in metastasis. It has been proposed (*40*) that induction of metastasis requires at least two gene complementation groups. In the correct recipient cells, one of these genes may be the ras oncogene. The other gene complementation group is not known, and there is no evidence for or against the possibility that it includes a metastasis suppressor gene that must be inactivated to permit full expression of ras.

The best evidence that ras-induced metastasis may be a balance of stimulatory and inhibitory factors comes from the study of Pozzatti et al. (*122*), who reported that second-passage (diploid) rat embryo fibroblasts transfected with ras alone were highly metastatic, but that rat embryo fibroblasts cotransfected with ras and a second oncogene, the adenovirus type 2 E1a gene, were virtually nonmetastatic, despite the fact that the cotransfected cells were tumorigenic. In this model system, transfection of the E1a alone did not result in transformed cells. The mechanism by which the E1a gene may have some inhibitory effect on the metastatic phenotype is not clear. However, Cook et al. (*123*) reported that the isolated expression of E1a was sufficient to cause increased cytolytic susceptibility, irrespective of histocompatibility antigen identity between killer cells and target cells in a hamster system. Whether or not immune cell lysis susceptibility is altered in the cotransfected rat embryo fibroblasts has not been determined. However, Garbisa et al. (*57*) demonstrated that the E1a suppression of metastasis in the cotransfected rat embryo fibroblasts was associated with a loss of type IV collagenolytic activity, and Steeg et al. (*124*) reported that expression of the NM23 gene associated with low tumor metastatic potential is increased during adenovirus 2 E1a inhibition of metastasis in cotransfected rat embryo fibroblasts. Thus, the E1a gene may indeed have intrinsic metastasis-inhibitory activity. This hypothesis is particularly intriguing in light of the recent observation that the tumor suppressor retinoblastoma gene product can form a specific complex with the E1a-transforming protein of adenovirus (*24*). However, in the rat embryo fibroblast system, Pozzatti et al. (*122*) found that E1a alone did not have transforming activity. Depending on cell type, the gene product of E1a may be able to complex with different proteins, which determine whether it functions as a transforming factor or as a metastasis suppressor.

RFLP analysis has also provided evidence for the existence of genes that specifically suppress the metastasis of specific tumors. Allelic deletions are common in metastatic colorectal cancers, and patients with more than the median percentage of allelic deletions have a worse prognosis than do other patients (*125*). Allelic deletions on chromosome 11 are associated with the increased aggressiveness of human breast cancer cells (*32*). The evidence for the association of ras and L-myc RFLPs with metastasis was reviewed above.

The selection of specific genes that are differentially expressed in metastatic versus nonmetastatic tumor cells has also been used to identify metastasis suppressor genes. Thus, c-fos may qualify as a metastasis suppressor gene, since its levels are increased in nonmetastatic tumor variants (*89*). The mechanism of action of c-fos may be via the up-regulation of the H-2K gene in murine tumor cells. The role of c-fos in human metastatic disease is unclear.

Dear et al. (*126*) used the technique of subtractive hybridization to isolate a cDNA clone of a novel gene, WDNM1, which is expressed to a greater degree in nonmetastatic rat mammary adenocarcinoma cells than in metastatic clones derived from the same parent line. It is not yet clear if differential expression of this

gene is associated with metastatic potential in other model systems.

Expression of another gene, NM23, which was originally isolated by the differential screening of a cDNA library, has been demonstrated to be differentially expressed in more than one metastasis model system (*124,127–129*). Originally isolated from a cDNA library of murine melanoma cells, the levels of the NM23 mRNA transcript were shown to vary with the metastatic potential of seven related murine melanoma cell lines; the highest NM23 RNA levels were in cells of relatively low metastatic potential (*127*). The levels of NM23 RNA did not correlate with cell sensitivity to host immunologic responses, and presumably, they are associated with intrinsic aggressiveness or lack thereof. It was subsequently shown that NM23 RNA levels were differentially expressed in another experimental system, *N*-nitroso-*N*-methylurea-induced mammary carcinomas in rats. In this system, levels of NM23 were highest in nonmetastatic tumors, intermediate in the primary tumors with metastatic potential, and lowest in pulmonary metastases (*127,128*). Mouse mammary tumor virus-induced mammary tumors of different metastatic potential also differentially express NM23 (*128*). As described above, NM23 RNA levels were also inversely correlated with the metastatic potential of ras-transfected rat embryo fibroblasts and the nonmetastatic ras plus E1a cotransfectants (*124*). It is not clear whether the NM23 gene mediates the E1a-induced inhibition of metastasis in the rat embryo fibroblast system or whether it is merely associated with it. Low levels of NM23 RNA have also been correlated with more highly metastatic breast cancers in human patients (*129*). Thus, the loss of NM23 gene expression is associated with metastasis in human as well as rodent systems and in more than one type of tumor. Sequence homology between the proteins encoded by NM23 and the *Drosophila* abnormal wing disc (awd) gene has been noted (*130*). Since awd mutations cause abnormal development, it is interesting to speculate that cancer metastasis may be linked to the loss of genes that normally regulate development.

Conclusions

It is now generally accepted (*11*) that there are loci in normal cells that can suppress the tumorigenic phenotype and that can be inactivated by mutation. Cancer cells must be tumorigenic to grow as a metastatic colony; however, all tumorigenic cells are not necessarily invasive and metastatic. The metastatic phenotype is independent from the tumorigenic phenotype (*10,84*). It is proposed that multiple gene products are necessary for the expression of the metastatic phenotype. Genetic control of metastasis may be exerted by the increased expression of specific genes involved in the metastatic cascade.

This review discussed the possibility that genetic control of metastasis is also exerted via the deactivation of specific genes, metastasis suppressor genes, in a manner analogous to the action of tumor suppressor genes on tumorigenicity. The evidence for the existence of metastasis suppressor genes is derived from somatic cell fusion studies, DNA transfection experiments, RFLP analysis, and the isolation of cDNA clones by subtractive hybridization or differential screening. The E1a gene of adenovirus has been shown in cotransfection experiments to inhibit the metastatic phenotype of ras oncogene-transfected rat embryo fibroblasts. The biological relevance of this finding is not clear, since the E1a gene is not a cellular gene. The c-fos gene and two other (nononcogene) cellular genes have been identified whose expression is augmented in nonmetastatic versus metastatic cells. The expression of one of these genes, NM23, has been demonstrated to be inversely correlated with metastasis in a variety of experimental model systems, as well as in human breast carcinomas. Definitive assessment of NM23 and other genes such as WDNM1 must await the identification and characterization of their gene products and demonstration of their ability to inhibit metastasis when they are transfected into cells that manifest the complete metastatic phenotype.

By analogy with the recessive tumor suppressor genes, one may hypothesize that a set of recessive metastasis suppressor genes exists that is susceptible to mutational inactivation. Two possible scenarios can be proposed. When metastasis suppressor genes are inactivated subsequent to the loss of tumor suppressor genes, the metastasis may be a late occurrence in tumor development. However, metastasis suppressor genes may also be inactivated prior to the loss of tumor suppression. Such inactivation or loss would be cryptic until the loss of tumor suppressor genes is accomplished. In such cases, metastatic disease may be an early event in tumor evolution. The identification of metastasis suppressor genes and the development of diagnostic tools for their detection may play a major role in the prognostic evaluation of cancers in the future.

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