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Gene products which play a role in cancer invasion and metastasis

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Summary

Invasion requires a number of distinct tumor cell interactions with host tissue, beginning with attachment to the matrix, followed by hydrolysis of matrix material and locomotion. Gene products which may be involved in these steps are discussed here. Laminin receptors and integrins have roles in the adhesion phase, while certain collagenases are prominent among the matrix-degrading enzymes. Autocrine motility factors, distinct from growth factors, appear to be involved in tumor cell locomotion. Finally, certain oncogenes, partricularly of the *ras* family, are closely related with metastatic potential. A detailed understanding of the molecular biology of invasion and metastasis could ultimately lead to specific means of interfering with or even reversing these malignant processes.

Multistep cascade of metastases

A metastatic colony is the end result of a complicated series of tumor host interactions (Table 1). Primary tumor initiation and progression is followed by the transition from *in situ* to locally invasive cancer and angiogenesis [1–6]. Newly formed tumor vessels are often defective and easily invaded by tumor cells within the primary mass. At the invasion front, tumor cells also invade pre-established host blood vessels. Tumor cells are discharged into the venous drainage in single cell form and in clumps. For rapidly growing tumors 1 cm in size, millions of tumor cells can be shed into circulation every day. Fortunately for the patient, only a very small percentage (<0.01%) of circulating tumor cells initiate metastatic colonies. Tumors generally lack a well-formed lymphatic network. Therefore, communication of tumor cells with lymphatic channels occurs only at the tumor periphery and not within the tumor mass. Tumor cells entering the lymphatic drainage are carried to regional lymph nodes where they arrest in the large lymphatics of the subcapsular sinus. Within 10 to 60 min after initial arrest in the lymph node, a significant fraction of the tumor cells detach and enter the afferent lymphatics. These tumor cells eventually end up in the regional or systemic venous drainage due to the existence of numerous lymphatic-hematogenous communications. Thus, the regional lymph node does not function as a true mechanical barrier to tumor dissemination. Lymphatic and hematogenous dissemination occurs in parallel.

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Tumor cell interaction with the extracellular matrix

The mammalian organism is composed of a series of tissue compartments separated from each other by two types of extracellular matrix: basement membranes and interstitial stroma [4]. The matrix determines tissue architecture, has important biologic functions, and exists as a mechanical barrier to invasion. During the transition from *in situ* to invasive carcinoma, tumor cells penetrate the epithelial basement membrane and enter the underlying interstitial stroma. Once the tumor cells enter the stroma they gain access to lymphatics and blood vessels for further dissemination. Fibrosarcomas and angiosarcomas, developing from stromal cells, invade surrounding muscle basement membrane and destroy myocytes. Tumor cells must cross basement membranes to invade nerve and most types of organ parenchyma. During intravasation or extravasation, the tumor cells of any histologic origin must penetrate the subendothelial basement membrane. In the distant organ where metastasis colonies are initiated, extravasated tumor cells must migrate through the perivascular interstitial stroma before tumor colony growth occurs in the organ parenchyma. Therefore, tumor cell interaction with the extracellular matrix occurs at multiple stages in the metastatic cascade.

General and widespread changes occur in the organization, distribution, and quantity of the epithelial basement membrane during the transition from benign to invasive carcinoma [5, 6]. The human breast is a particular example. Benign proliferative disorders of the breast such as fibrocystic disease, sclerosing adenosis, intraductal hyperplasia, fibroadenoma, and intraductal papilloma are all characterized by disorganization of the normal epithelial stromal architecture. Extreme forms can mimic the appearance of invasive carcinoma. However, no matter how extensive the architectural disorganization, these benign disorders are always characterized by a continuous basement membrane separating the epithelium from the stroma. In contrast, invasive ductual carcinoma, invasive lobular carcinoma, and tubular carcinoma consistently possess a defective extracellular basement membrane, with zones of basement membrane loss around the invading tumor cells in the stroma. The basement membrane is also markedly defective adjacent to tumor cells in lymph node and organ

Table 1.

Metastatic cascade event	Potential mechanisms
1. Tumor initiation	Carcinogenic insult, oncogene activation or derepression, chromosome rearrangement
2. Promotion and progression	Karyotypic, genetic, and epigenetic instability, gene amplification; promotion-associated genes and hormones
3. Uncontrolled proliferation	Autocrine growth factors or their receptors, receptors for host hormones such as estrogen
4. Angiogenesis	Multiple angiogenesis factors including known growth factors
5. Invasion of local tissues, blood and lymphatic vessels	Serum chemoattractants, autocrine motility factors, attachment receptors, degradative enzymes
6. Circulating tumor cell arrest and extravasation	Tumor cell homotypic or heterotypic aggregation
a. adherence to endothelium	tumor cell interaction with fibrin, platelets, and clotting factors; adhesion to RGD type receptors
b. retraction of endothelium	platelet factors, tumor cell factors
c. adhesion to basement membrane	laminin receptor, thrombospondin receptor
d. dissolution of basement membrane	degradative proteases, type IV collagenase, heparanase, cathepsins
e. locomotion	autocrine motility factors, chemotaxis factors
7. Colony formation at secondary site	Receptors for local tissue growth factors, angiogenesis factors
8. Evasion of host defenses and resistance to therapy	Resistance to killing by host macrophages, natural killer cells and activated T cells; failure to express, or blocking of, tumor specific antigens; amplification of drug resistance genes

metastases. In some focal regions of well differentiated carcinoma, partial basement membrane formation by differentiated structures can be identified. These findings are of direct application to diagnostic problems in surgical pathology such as the differentiation of tangential sections of *in situ* lesions from true invasion, or of differentiating severe adenosis from invasive carcinoma. Loss of basement membranes in human carcinomas significantly correlates with increased incidence of metastases and poor 5-year survival.

Three-step theory of invasion

A three-step hypothesis has been proposed for describing the sequence of biochemical events during tumor cell invasion of extracellular matrix [4]. The first step is tumor cell attachment to the matrix. Attachment may be mediated by specific glycoproteins such as laminin and fibronectin through tumor cell plasma membrane receptors. Following attachment, the tumor cell secretes hydrolytic enzymes (or induces host cells to secrete enzymes) which can locally degrade the matrix (including degradation of the attachment glycoproteins). Matrix lysis most likely takes place in a highly localized region close to the tumor cell surface, where the amount of active enzyme outbalances the natural protease inhibitors present in the serum and in the matrix itself. In contrast to the invasive tumor cell, when the normal cell or benign tumor cell attaches to the matrix it may respond by shifting into a resting or differentiated state. The third step is tumor cell locomotion into the region of the matrix modified by proteolysis. The direction of the locomotion may be influenced by chemotactic factors and autocrine motility factors. Autocrine motility factors (AMF) are a newly described class of proteins which bind to a cell surface receptor and profoundly stimulate motility. They are distinct from known growth factors, and their mechanism of action involves the membrane G protein pathway inhibited by pertussis toxin. The chemotactic factors derived from serum, organ parenchyma, or the matrix itself may influence the organ specificity of metastases. Continued invasion of the matrix may take place by cyclic repetition of these three steps.

Laminin receptors

Cell surface receptors for the basement membrane glycoprotein laminin mediate adhesion of tumor cells to the basement membrane prior to invasion [7–9]. Laminin as visualized by rotary shadowing electron microscopy has a distinctive cruciform shape with three short arms (35 nm) and one long arm (75 nm). All arms have globular end regions. The specialized structure of the laminin molecule may contribute to its multiple biologic functions. Laminin plays a role in cell attachment, cell spreading, mitogenesis, neurite outgrowth, morphogenesis, and cell movement. Many types of neoplastic cells contain high affinity (nM Kd) cell surface binding sites (laminin receptors) for laminin. The molucular weight of the isolated receptor is 65 kDa [7]. The laminin receptor binds to the 'B' chain (short arm) region of the laminin molecule. Laminin receptors may be altered in number or degree of occupancy in human carcinomas. This may be the indirect result of defective basement membrane organization in the carcinomas. Breast carcinoma and colon carcinoma tissue contains a higher number of exposed (unoccupied) receptors compared to benign lesions. The laminin receptors of normal epithelium may be polarized at the basal surface and occupied with laminin in the basement membrane. In contrast, the laminin receptors on invading carcinoma cells are amplified and may be distributed over the entire surface of the cell. The laminin receptor can be shown experimentally to play a role in hematogenous metastases [10]. Treating tumor cells with the receptor-binding fragment of laminin at very low concentrations markedly inhibits or abolishes lung metatases from hematogenously introduced tumor cells by blocking the adhesion of circulating tumor cells to the subendothelial basement membrane.

RGD recognition receptors

A family of cell surface glycoproteins termed 'integrins' has been identified which bind with low affinity (μM Kd) to a variety of adhesion proteins including fibronectin, von Willebrand factor, fibrin, vitronectin, type I collagen, and thrombospondin. The integrins are a complex of alpha (140 kDa) and beta (95 kDa) subunit proteins. The functions of several of the integrins are inhibited by peptides related to the Arg-Gly-Asp (RGD) sequence of fibronectin. RGD sequences present in a wide variety of proteins may serve as the recognition site for binding of the integrins. It is likely that specific ligand sequences adjacent to the RGD site may confer preferential recognition of one type of adhesion protein by certain members of the integrin family. Integrin proteins are thought to align adhesion proteins such as fibronectin on the cell surface with cytoskeletal components such as talin and actin, thus altering cell shape. Integrin type proteins may play an adhesive role in platelet-tumor interactions, binding of lymphoid cells to endothelium, and interaction of circulating tumor cells with endothelial surfaces, fibrin, von Willebrand factor, or thrombospondin. In keeping with this concept, it has been reported that co-injection of tumor cells with large quantities of RGD peptides will inhibit metastasis formation in animal models [12]. The RGD peptides may interfere with the adhesion of tumor cells to the endothelial surface; this action may directly or indirectly be mediated through integrin proteins.

Tumor cell proteinases

In vitro studies of tumor cell invasion of the extracellular matrix have shown that cell proliferation is not absolutely required [13]. Invasion of the matrix is not merely due to passive growth pressure, but requires active biochemical mechamisms. Inhibitors of protein synthesis or inhibitors of proteinases block invasion of the matrix [1]. Many research groups have proposed that invasive tumor cells secrete matrix-degrading enzymes [14–20]. Collagen is an important substrate because it constitutes the structural scaffolding upon which the other components of the matrix are assembled. Tumor-derived collagenases which degrade interstitial collagen types I, II and III have been characterized by a number of investigators. They are metal ion (calcium and zinc) dependent enzymes which function at neutral pH [15, 16]. Classic collagenase produces a single cleavage in the collagen molecule (interstitial collagen types I, II, and III) producing 3/4 and 1/4 size fragments (75% of the distance from the N terminus). Tumor cells can degrade both collagenous and non-collagenous components of the basement membrane. Basement membrane specific collagen types IV and V are *not* susceptible to classic collagenase which degrades collagen types I, II, and III. A separate family of collagenolytic enzymes (type IV collagenase) cleave the type IV collagen chain 1/4 of the distance from the amino terminus [18, 19]. Type IV collagenases are augmented in highly metastatic tumor cells and in endothelial cells during angiogenesis. Antibodies prepared against type IV collagenase react with invading breast carcinoma cells and breast carcinoma lymph node metastases by immunohistology. Amplification of type IV collagenase production is biochemically linked to the genetic induction of metastases in experimental models [25, 37].

Molucular genetics of metastases

It is apparent that interactions in the complicated metastatic process involve multiple gene products. A cascade or coordinated group of gene products expressed above a certain threshold level may be required for a tumor cell to successfully traverse the successive steps in the metastatic process. The crucial gene products may regulate host immune recognition of the tumor cells, cell growth, attachment, proteolysis, locomotion, and differentiation. The specific family of gene products necessary for metastases may be different for each histologic type of tumor.

A growing list of transforming genes or 'oncogenes' have been identified which may be involved in the genetic alterations leading to tumor growth, invasion, and metatases [21–24]. Following intro-

duction into suitable recipient cells, oncogenes confer anchorage independent colony growth in soft agar, and in many cases, tumorigenicity in animal hosts. Cancer cells must, of course, be tumorigenic in order to grow as a metastatic colony. However, all tumorigenic cells are not necessarily invasive and metastatic. This is because the metastatic phenotype is independent from the tumorigenic phenotype. Most of the past work on oncogene function has emphasized mechanisms related to alteration of growth control. Investigators studying oncogene-transformed cells rarely tested these cells for the ability to produce metastases in animal models.

Now it has been recognized that some oncogene classes can induce the complete metastatic phenotype in the appropriate recipient cell [25–35]. An important example is the *ras*^{H} oncogene. Transfection of members of the *ras* family of oncogenes into rat or into mouse embryo derived fibroblasts [25, 26, 29, 34] will lead to full expression of the metastatic phenotype. The mechanism of metastasis induction by *ras* is not related to changes in sensitivity to killing by immune cells. Metastatis induction by *ras* is associated with a cascade of gene expression which elevates the intrinsic aggressiveness of the tumor cells.

Thorgeirsson *et al.* [25] were the first to report the metastatic propensity of murine cells transformed with human tumor genomic DNA. Mouse embryo derived fibroblasts (NIH-3T3 cells) transfected with AML or bladder cancer tumor DNA produced numerous metastases when injected into immunodeficient nude mice.

When the resultant metastatic clones were examined, they were found to have acquired exogenous activated *ras* oncogene sequences. In order to test whether the *ras* oncogene itself or associated genomic DNA was responsible for the metastatic induction, cloned defined *ras* oncogenes were transferred into NIH-3T3 cells. The resultant *ras* transformed cells were fully metastatic but had not become resistant to NK cell or macrophage lysis. *Ras* transfected cells also produce metastases in a non-mammalian system, the chick embryo, as reported by Bondy *et al.* [27].

Muschel *et al.* [26, 33] transformed NIH-3T3 cells with the viral *ras*^{H} oncogene or the *ras*^{H} oncogene from the T24 human bladder carcinoma cell line and isolated multiple independent clones. All of the clones were metastatic following injection into nude mice. Egan *et al.* [29] confirmed these results and found that the number of lung metastases produced was proportional to the level of the *ras* oncogene encoded P21 protein in each transformant. The rare metastatic variants isolated from cells with barely detectable *ras*^{H} were found to have high levels of *ras* expression caused by rearrangement or amplification at the DNA level. Egan *et al.* [9] also used a steroid responsive promoter to show the importance of *ras* oncogene transcript dose on metastasis production. The level of *ras* expression in these experimental models correlates directly with metastatic potential. The *ras*^{H} oncogene is distinguished from its normal cellular counterpart by one or more point mutations [22]. In the viral and T24 *ras*, the mutation is at the position coding for the 12th amino acid. Transfection of the normal proto-oncogene lacking the mutation will not cause transformation. However, Chang *et al.* [23] demonstrated that the *ras* proto-oncogene joined to a viral promoter and transcriptional enhancer would cause elevated production of the normal P21 protein and could transform NIH-3T3 cells. The cells transformed with elevated levels of the normal *ras* (encoding normal P21 protein) produced tumors at a rate comparable to cells transformed with the mutated *ras* [23], but when the same cells were tested for metastatic propensity, the cells transformed by the mutated *ras* were much more efficient in the production of metastases [26, 29]. Nevertheless, very high levels of the normal P21 could also lead to metastasis production. Taken together, all the results are consistent with a dominant role for *ras*-encoded protein dose in the induction of metastases. Very low levels of the mutated P21 protein will result in poorly metastatic tumors, and moderate to high levels of mutated P21 protein will result in highly metastatic tumors. In contrast, low or moderate levels of the normal P21 protein will not result in tumorigenicity. However, high levels of normal P21 will result in tumors and very high levels of P21 will produce metastatic tumors.

The use of NIH-3T3 cells in experimental models

of metastasis induction by oncogenes has been criticized because these cells are aneuploid and have a high rate of spontaneous transformation [28]. It was conceivable that *ras* oncogene induction of metastases might require cellular or genetic properties only present in NIH-3T3 cells. Therefore, Muschel *et al.* [26] and Pozzatti *et al.* [34] tested the metastatic propensity of *ras*-transfected diploid primary cells. Muschel *et al.* [26] found that rat skin cells, rat muscle cells, and Chinese hamster lung fibroblasts were induced to become metastatic by transfection of *ras* linked to an enhancer using a construct of Spandidos and Wilkie. Pozzatti *et al.* [34] examined a series of diploid rat embryo cell clones which had been transformed by ras^H alone or ras^H linked to an SV-40 enhancer cotransfected with a dominant selectible marker (pRSVneo). These clones were all highly metastatic following intravenous, subcutaneous, or intramuscular injection into nude mice. Thus, the ability of the ras^H oncogene to induce metastases is not limited to NIH-3T3 cells but occurs even after transformation of certain diploid primary cells.

The *ras* oncogene can also amplify the metastatic potential in certain low or nonmetastatic established tumor cell lines which were not originally transformed by ras^H. Vousden *et al.* [30] transfected ras^H into a highly tumorigenic cell line derived from a murine mammary carcinoma. While the parent cell line was very weakly metastatic, the subclones transfected with *ras* were all highly metastatic. Multiple clones were isolated from the resulting lung metastases, and most retained the metastatic phenotype. One of the clones was no longer metastatic, and it was found to have lost the introduced *ras* oncogene. Collard *et al.* [12] obtained similar results when *ras* was inserted into T lymphoma cells; the lymphoma cells became invasive and metastatic to a degree proportional with the level of ras^H specific mRNA. Kerbel *et al.* [31] had similar results with SP1, a cell line isolated from a nonmetastatic murine mammary carcinoma. Metastatic primary tumors were produced by all clones which incorporated the mutated but not the normal *ras*.

The ability of the ras^H oncogene to induce metastases is dependent on the cell type. Transfection of ras^H into Cl27 murine cells will result in highly tumorigenic cells which fail to form metastases following intravenous or subcutaneous injection into nude mice. The carcinogen N-nitrosomethylurea will induce skin papillomas and mammary tumors [35, 36] in appropriate strains of mice. The vast majority of the induced tumors have an activated *ras* oncogene [36], but are nonmetastatic. Likewise, only 10% of DMBA tumors will produce metastases. These results lead to the conclusion that activation of the ras^H oncogene is not sufficient to induce metastases in certain cultured cell types or spontaneous tumors. The *ras* oncogene may fail to induce metastases in a particular cell because that cell lacks an appropriate cooperation factor. On the other hand, the resistant cell may contain a means of suppressing the ability of *ras* to induce metastases. The adenovirus 2 E1A oncogene is an example of a gene which can suppress metastasis caused by ras^H. Pozzatti *et al.* [34] showed that cotransfection of E1A with *ras* will result in nonmetastatic tumors. The mechanism of inhibition may involve the 12S E1A transcript, and is not related to histo-compatibility antigen changes or increased sensitivity to immune cell killing. The results lead us to predict the existence of normal genes which function to suppress the metastatic cascade induced by certain oncogenes such as *ras*.

The mechanism by which the ras^H oncogene can induce metastases in the appropriate cell recipient is unknown. It must involve the activation of a multigene cascade, since the ras^H transformed cells acquire a large number of new functional properties including increased adhesiveness, motility, and ability to invade tissue barriers [25, 37–39]. One potential explanation is that ras^H transfection leads to genetic or karyotypic instability with the resultant selection of metastatic variants [24]. A second possible mechanism could involve the selected integration of ras^H into a specific location in the genome next to metastasis-associated genes. Both of these explanations seem unlikely based on the data obtained so far. Diploid rat embryo fibroblasts transfected with ras^H become metastatic as soon as enough cells (4 passages) can be grown up to inject into nude mice. The resulting metastatic clones do not contain any consistent gross karyotypic alter-

ation and indeed may remain full diploid [33]. All of the transfectant clones expressing the activated P21 protein are metastatic [26, 29, 34], implying that induction of metastases is not a rare event as would be expected if there was a requirement for *ras* to be integrated into a specific site. The explanation we are left with is that the *ras* P21 protein alters some general pathway in the cell and that this pathway is involved in the metastatic cascade. A likely candidate pathway is the G protein mediated transducer systems involved in phosphatidylinositol-4,5-bisphosphate and catabolites thereof, as well as the arachidonic acid pathways mediated through phospholipase A2 [39–41].

Ras^{H} is not the only oncogene which can induce metastatic potential in 3T3 cells. Egan *et al.* [41] recently reported that certain transforming oncogenes encoding protein kinases (*mos, raf, src, fes,* and *fms*), but not nuclear oncogenes such as *myc* or p53, induced 3T3 cells to produce lung metastases following intravenous injection. Whether or not these oncogenes will induce the metastatic phenotype in diploid primary cells is, as yet, unknown. Transformation of cells by *src, fes,* and *fms* may be mediated through a *ras*-dependent mechanism, since Smith *et al.* [42] have shown that transformation by these oncogenes is blocked by antibodies to *ras*-encoded P21. Oncogenes have been established to have multifactorial effects on a variety of general cell pathways [43, 44].

Regardless of the mechanism by which oncogene transfection can induce metastases in animal systems, it constitutes a revolutionary model system for studying the biochemical mechanisms of metastasis. For example, specific classes of collagenase [35] and motility-stimulating cytokines [40] have been shown to be biochemically linked to the induction of metastases by the *ras*^{H} oncogene. Using appropriate combinations of *ras*^{H} oncogenes with viral enhancers or other oncogenes such as E1A, diploid cells will become a) fully tumorigenic but non or low metastatic, or b) fully metastatic. The metastatic clones are very aggressive, producing more than 200 metastases in the lungs of nude mice following intravenous injection of only 5×10^4 cells. A wide variety of organs are the site of spontaneous metastases produced from primary tumors arising from subcutaneous or intramuscular injection of transfected cells. Virtually unlimited numbers of clones of metastatic or nonmetastatic tumor cells can be produced using transfection methods. The transfection model system is vastly superior to previous metastasis models which were the result of multiple transplantation selection steps applied to heterogeneous transplantable tumors [24].

Oncogene expression correlation with human tumor metastatic aggressiveness

Proto-oncogenes may be activated and may contribute to neoplastic transformation and progression to the metastatic phenotype [21–23; 45–54]. Activation can occur by multiple pathways, including a) amplification of the number of copies of the oncogene in the genome of tumor cells, b) mutation within the coding sequence of the oncogene, c) chromosomal breaks and translocation with subsequent enhanced expression of the oncogene encoded protein, and d) insertion of a retroviral promoter near the proto-oncogene. Yokota *et al.* [33] studied proto-oncogene alteration in 72 samples of tumor tissue and corresponding normal tissue from the same patient. Alterations were frequently found in c-*myc,* c-*ras*, and c-*myb*. No oncogene alterations were observed in the normal tissue. Oncogene alterations may merely be a hallmark of the genetic instability of tumors. On the other hand, if proto-oncogene alterations play an actual functional role in the malignant behavior, they might provide a survival advantage and be selected for in the expanding tumor cell population. This could result in the increased expression of relevant oncogene products in more aggressive tumors with a higher propensity for metastases. In fact, oncogene expression is found to be correlated with the metastatic behavior in certain classes of human tumors studied to date. However, a different class of oncogene appears to be important for each histologic type of tumor.

Amplification of the HER-2/*neu* oncogene has been correlated with metastases in human breast carcinoma. The HER-2/*neu* (*neu*) oncogene is a member of the *erb* B-like oncogene family, and is

related to, but distinct from, the gene encoding the epidermal growth factor receptor. Slamon *et al.* [46] studied alterations in the gene in 189 primary human breast cancer specimens; *neu* was amplified in 30% of the tumors. Amplification was a significant predictor of overall survival, time to relapse, estrogen receptor status, size of primary tumor, as well as the number of axillary lymph nodes positive for metastases. Van der Vijver *et al.* [48] detected amplification of *neu* in 16 of 95 human breast tumor samples and this was accompanied by overexpression in the tumors in which intact RNA could be isolated. No correlation was found in this study between *neu* amplification and estrogen receptor content, age, or clinical stage of disease.

Increased expression of the Ha-*ras* oncogene has also been correlated with lymph node metastases in human breast carcinoma. Agnantis *et al.* [49] found a significant elevation of Ha-*ras* transcripts in malignant compared to normal breast tissue, with a higher mean value of expression in cases with lymph node metastases. Hand *et al.* [51] immunologically assayed the Ha-*ras* P21 protein in samples of human breast carcinoma and colon carcinoma. Enhanced expression was documented in 66% of breast and 100% of colon carcinomas compared to normal counterparts, with levels in breast carcinoma ranging from 18.4 to 51.7 pg P21/μg protein. Clair *et al.* [50] extended this finding to report a correlation of breast carcinoma P21 expression with advanced disease stage and positive axillary lymph node metastases. Lundy *et al.* [52] reported a positive correlation between Ha-*ras* P21 protein levels and lymph node metastases, but not patient age or estrogen receptor status.

N-*myc* amplification is associated with rapid progression of neuroblastomas. Seeger *et al.* [54] studied 89 patients with untreated primary neuroblastoma to determine the relation between the number of copies of the N-*myc* oncogene and survival without disease progression. Analysis of progression-free survival in all patients revealed that amplification of N-*myc* was associated with the worst prognosis. The estimated progression-free survival at 18 months was 70%, 30%, and 5% for patients whose tumors had 1, 3 to 10, or more than 10 N-*myc* copies. It is unclear whether or not the poor survival in patients with amplified N-*myc* is due to an increased number of metastases. However, amplified N-*myc* is prevalent in stage 4 neuroblastomas [55] which have distant metastases from hematogenous or lymphatic dissemination. The mechanism by which N-*myc* augments tumor aggressiveness is unknown. Experimental animal studies to date have not shown a significant role for N-*myc* transfection (N-*myc* alone or in combination with H-*ras* [37, 41]) in the induction of the metastatic phenotype. However, these experiments have not been conducted with neural cell lines. Patients whose neuroblastoma tumor cells can be grown *in vitro* as a cell line have a very high association with amplified N-*myc* and poor prognosis. Thus, it is conceivable that N-*myc* amplification somehow facilitaties the independent growth of neuroblastoma cells in a harsh environment. This would certainly favor the growth of metastatic colonies in distant organ sites. Neuroblastoma cells without N-*myc* amplification may have a greater requirement for cooperating local host factors which support growth.

Tumors other than neuroblastoma have not shown as strong a correlation between N-*myc* amplification and clinical prognosis or extent of metastases. In contrast to *neu* amplification and H-*ras* overexpression, C-*myc* or N-*myc* oncogene amplification was not correlated with human breast cancer stage of disease, hormonal receptor status, or axillary lymph node metastases [45, 46]. C-*myc* and N-*myc* are amplified in small cell lung cancers and gastrointestinal malignancies, but the level of amplification has not been shown to correlate with metastases [56, 57]. Thus, if oncogenes are indeed important in human tumor progression, the effect of any given oncogene may depend on the genetic background of the host cell.

Tumor cell motility factors

Cell motility is necessary for tumor cells to traverse many stages in the complex cascade of invasion. Such stages could include the detachment and subsequent infiltration of cells from the primary tumor into adjacent tissue, the migration of the cells through the vascular wall into the circulation (in-

travasation), and the extravasation of the cells to a secondary site. The movement of cells through biologic barriers such as the endothelial basement membranes of the vasculature may well occur by means of chemotactic mechanisms. Indeed, studies on *in vitro* chemotaxis of some tumor cells indicate that a variety of compounds such as complement-derived materials, collagen peptides, formyl peptides, and certain connective tissue components can act as chemoattractants [58, 59].

While these agents may well contribute to the directional aspects of a motile response, they are not sufficient to initiate the intrinsic locomotion of tumor cells. The availability of soluble attractants to the tumor cell is greatly dependent upon the host, even in those cases in which the production of attractants is the result of tumor cell-host tissue interaction. At best, it seems that the cell would have access to such motility stimuli at sporadic and irregular intervals. Such conditions are unfavorable to a sustained migration of highly invasive cells.

With these considerations in mind and stimulated by the studies of Anzano *et al.* [60], in which they demonstrated autocrine growth factors for transformed cells, we investigated the possibility that such cells could elaborate autocrine motility factors. The action of these substances might, in part, explain both the markedly invasive character and the metastatic property of malignant neoplastic cells. Thus, under the influence of such an autocrine material, a tumor cell might move out into the surrounding host tissue and also exert a 'recruiting' effect on adjacent tumor cells in the presence of a gradient of attractant. Conceivably, such factors might also attract fibroblastic cells of the host, resulting in the phenomenon of desmoplasia, characteristic of invasive tumors.

We have found that the human melanoma cell line A2058 and human breast carcinoma cells produce in culture a material that markedly stimulates their own motility [40, 60, 61]. These cells respond in a dose-dependent manner to various concentrations of conditioned medium obtained by incubating confluent cells in serum-free medium an indication that the motility factor is derived from the cell. Motility was measured by the modified Boyden chamber procedure. Using this assay and the 'checkerboard' analysis [40], we have also found that the conditioned medium factor has both chemotactic (directional) and chemokinetic (randomly motile) properties.

Early events in migration may involve pseudopodia protrusion [61]. During the course of invasion, the same tumor cell must interact with a variety of extracellular matrix proteins as it traverses each tissue barrier. For example, the tumor cell encounters laminin and type IV collagen when it penetrates the basement membrane, and type I collagen and fibronectin when it crosses the interstitial stroma. It has recently been shown that cells express specific cell surface receptors which recognize extracellular matrix proteins. The first example of such a receptor is the laminin receptor, which binds to laminin with a nanomolar affinity. Laminin receptors have been shown to be augmented in actively invading tumor cells, and may play an important role in tumor cell interaction with the basement membrane. Arg-Gly-Asp (RGD) recognition receptors are another class of cell surface proteins which bind extracellular matrix proteins which in turn contain the protein sequence arg-gly-asp [11]. Such proteins include fibronectin, collagen type I, and vitronectin. The process of cell migration undoubtedly requires a series of adhesion and detachment steps resulting in traction and propulsion. Studies using AMF-stimulated motility as model system have revealed an important function of pseudopodia protrusion in this process. AMF stimulates motility on a variety of different substrata. Therefore, its action is independent of the mechanism of attachment. Furthermore, AMF induces the rapid protrusion of pseudopodia in both a time and a dose-dependent manner [61]. Isolation of the induced pseudopodia reveals that they are highly enriched in their content of laminin and fibronectin matrix receptors [61]. Since cell pseudopodia formation is known to be a prominent feature of actively motile cells, we can now set forth a working hypothesis to explain the early events in cell motility. Cytokines such as AMF which stimulate intrinsic motility may induce exploratory pseudopodia prior to cell translocation. Such pseudopodia may express augmented levels of matrix

receptors (and possibly proteinases). The protruding pseudopodia may serve multiple functions including 1) acting as 'sense organs' to interact with the extracellular matrix proteins and thereby locate directional cues, 2) providing propulsive traction for locomotion, and 3) even inducing local matrix proteolysis to assist in the penetration of the matrix.

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