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# Tumor Cell Motility and Metastasis

*Autocrine Motility Factor as an Example of Ecto/Exoenzyme Cytokines*

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**Cellular locomotion plays a critical role in such normal processes as embryonic development, tissue segregation, as well as the infiltration of fibroblasts and vascular cells during wound repair and the inflammatory responses of the adult immune system. During tumor invasion and metastasis the processes of cell migration achieve dire significance. Disruption of normal homeostatic mechanisms to benefit the survival of the individual tumor cell is a common theme discovered during the characterization of factors once thought to be tumor-specific. One such molecule, tumor cell autocrine motility factor, was so described and has only recently been identified as a normal protein involved in intracellular glycolysis as well as implicated as an extracellular effector of normal cell functions including survival of certain populations of neurons. This molecule represents a member of the newly emerging family of intracellular enzymes whose disparate functions as extracellular mediators of cellular responses defines a new class of ecto/exoenzymes which play a role in normal cellular processes and are inappropriately utilized by tumor cells to elicit new survival strategies.** (Pathology Oncology Research Vol 3, No 3, 230–254, 1997)

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### *Introduction*

One characteristic and the most lethal feature of malignant tumors is their ability to metastasize, a process which involves tumor cell motility and the ability to invade host barriers. The metastatic process can be divided into sequential steps including: tumor cell detachment from the primary neoplasm and invasion of the extracellular matrix (ECM), intravasation of the hematogenous or lymph system, arrest in a secondary organ site, extravasation from the vessel of delivery, and proliferation in the newly defined site.[1] This cascade is not necessarily an end-point endeavor in that it can be repeated, which leads to the development of secondary and tertiary metastases (or metastases of metastases). Several steps in this cascade are dependent on the motility of the invading cells, specifically dissemination, intravasation and extravasation, and while random kinesis can play an important role in the detachment of cells from the primary tumor, directed motility, governed by gradients of soluble or fixed factors, is generally considered a prerequisite for tumor cell intravasation into a dissemination vessel as well as extravasation out of such vessels and into the target organ of secondary growth.

During the metastatic process tumor cells must infiltrate distinct interstitial ECM environments by crossing into tissue compartments which are often separated by basement membranes (BM's). These boundaries are considered to be the main barrier to tumor invasion, and it has been proposed that invasion of the BM and its associated connective tissue occurs in three steps: attachment, degradation and migration.[2] Numerous components of this pathway are required for a tumor cell to complete this invasive

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cascade, however the role of soluble factors in the extracellular millieu has been proposed as a variable which is often a limiting aspect to cellular invasive processes, including the induction of neovascularization by normal endothelium in response to angiogenic growth factors.

***Basic aspects of cellular motility***

*Mechanistic concerns*

The crawling movement of animal cells can manifest itself in different variations on a theme, however it is invariably based on the extension of a thin, wide leading lamella in the direction of migration.[3-7] Through the adherent lamella, tensional forces are generated which culminate in retraction of the trailing edge of the cell. These extension and retraction processes are not necessarily temporally distinct, but may be separated only spatially within the intact cell.[3] During *in vivo* migration events, where adhesion often occurs around the entire cell circumference, the leading portion of the cell is often cylindrical, resembling in vitro filopodia, however both lamellipodia and filopodia are lacking in intracellular organelles, presumably due to structural and/or spatial requirements of the motor protein components necessary to impart the requisite malleability to the extending protrusion.[8] Extension of the leading edge can be rationalized by simple osmotic or hydrostatic pressures, or as a result of the polymerization of filamentous actin itself, however the stabilization of the leading lamella depends in all cases on actin polymerization, during which the extending barbed ends of the actin filaments are oriented toward the advancing edge of the lamella.[3,4] The generation of newly available barbed ends for extension of filamentous actin can occur via uncapping of pre-existing filaments, the severing of these pre-existing filaments, or a combination of these processes, in addition to the possible *de novo* formation of actin nucleation sites.[9-11] It is interesting to note that both lamellipodia and filopodia contain interconnected extending filamentous actin, however their characteristic morphologies differ markedly and both processes can occur simultaneously at the same subcellular location within an isolated cell.[12] This phenomenon has been correlated with differential expression and localization of actin-binding proteins within these structures.[13]

As a result of forward movement, the cell must retract its trailing edge in order to once again enter the cycle of forward extension. The exertion of contractile force to achieve this retraction is thought to be mediated primarily by the motor protein myosin II.[14,15] Indeed it has been demonstrated that myosin II mutants exhibit decreased motility which is related to their inability to retract their trailing edge without apparent effect on the extension of the leading lamella.[16] Mechanistically speaking, the ATPase activity and organization of myosin II molecules into bipolar filaments is regulated by the calcium/calmodulin-dependent enzyme myosin light chain kinase (MLCK),[17] a system which enables distinct activation states of MLCK in specific subcellular regions as a result of differential $Ca^{2+}$ mobilization at the front or rear of the cell. Expression of myosin II is lower in the extending edge of the leading lamella, and the myosin II rods observed at this location appear disorganized and randomly oriented as compared to those at the base of this structure, where the filaments are oriented in parallel and arranged into ribbons.[15] The contractile force exerted by myosin II not only provides retraction of the trailing edge, but also aligns actin filaments into parallel bundles which are competent to develop into stress fibers as the cell becomes stationary.[14]

In order to retract the trailing edge, the cell must release the adhesive interactions which tethered the cell to the substratum and provided the capacity for initial extension of the leading edge. This process can be accomplished in several ways, including mechanical membrane ripping whereby adhesion molecules remain on the substratum.[18] This process can be induced by via inhibition of MLCK dephosphorylation which results in an elevated contractile state of the cell.[19] Similarly, transient increases in $Ca^{2+}$ concentrations at the rear of the cell which regulate the proper spatial activation of enzymes responsible for these contractile processes can also contribute to the release of trailing edge adhesions by activating actin-severing proteins which promotes the recirculation of actin monomers to the leading edge where they can be recycled for further use.[11]

A more regulated manner of trailing edge release has been suggested which relies invokes release of the cell's rear adhesions via intracellular signaling pathways. In one case, calcium transients activate the calcium/calmodulin-dependent serine/threonine phosphatase calcineurin (PP2B), causing the release of adhesive integrins.[20] Peptide inhibitors of calcineurin suppressed detachment of the trailing cell edge without effect on extension of the leading lamella, again stressing the independence of these two processes.[21] The GTP-binding protein rho appears to be a mediator of trailing edge release since inactivation of rho induces cytoskeletal breakdown which causes cell rounding and suppresses cell migration,[22,23] and tyrosine phosphorylation has also been suggested as a regulator of this process since ATP-induction of protein tyrosine phosphorylation destabilizes focal contacts and both the phosphorylation and the reorganization of adhesive components are suppressed by the introduction of exogenous tyrosine phosphatase to the system.[19] Obviously, the intracellular signaling processes which coordinate cell movement are complex and interwoven. It is not the purpose of this review to focus on this aspect of cellular locomotion, however the interconnected roles of calcium transients,

phosphoinositide metabolism, small GTP-binding proteins, protein kinases, and actin-regulating proteins in the mechanical processes requisite for achievement of cellular translocation are described in further detail elsewhere.[8,11]

*Extracellular matrix interactions in cell migration*

Cellular attachment to extracellular support structures allows the transduction of intracellular extension and contraction into cellular translocation along the cellular substratum. In this context, integrins play a critical role, and while high adhesive avidity of the matrix as well as elevated cell spreading and significant cellular focal adhesion plaques suppress motility, low cellular substrate adhesion can also impair motility by reducing the cell's ability to exert the tractional force necessary for movement.[5,24,25] This process depends on the concentration of the substrate on one hand, and on cellular adhesion molecules and cytoskeletal components on the other. It has been demonstrated that intermediate concentrations of substrate proteins promote maximal cell movement, with optimal motility-promoting concentrations being specific to each matrix component.[24,25] Similarly, integrin expression level can reciprocally affect cellular migration,[26,27] and elegant work by Palecek, Horwitz and colleagues recently demonstrated a complex regulatory network involving modulation of integrin expression, activation state, and extracellular substratum availability.[28] This report showed that the concentration of ECM ligand which promoted a maximal rate of cellular locomotion decreases reciprocally as integrin expression increases, while increasing integrin-ligand affinity yielded maximal migration at lower ligand concentrations, suggesting a maximal tractional force-generating level for each component of this system. Indeed, maximal migration speed remained unchanged as each variable fluctuated, further distinguishing integrin coupling with cellular motor molecules as the limiting factor in all cases. Together with the fact that overloading any component of this tripartite system (integrin expression, affinity or ligand concentration) resulted in decreased locomotion, these findings highlight the coordinate role these factors play in the concerted effort of cell migration within relevant matrices *in vivo*.

Although integrins are critically involved in cellular migration, other molecules influence the motile phenotypes of cells as well. For example, cadherins are known to mediate cell-cell homotypic interactions in a manner which regulates tissue morphogenesis.[29] Accordingly, the epithelial homotypic adhesion molecule E-cadherin has been shown to suppress the invasiveness of human carcinoma cells[30] and down-regulation of this molecule is associated with increased cellular motility and less favorable prognosis in several forms of cancer.[31] In a similar fashion, the focal contact-associated extracellular face heparan sulfate proteoglycan syndecan 4 mediates interaction of fibroblasts with the heparin-binding domain of fibronectin, a region which is required for the formation of stable cellular adhesions on this ECM molecule.[32] Of particular significance is the hyaluronic acid (HA)-binding cell surface receptor CD44, which has been implicated in cell-ECM adhesion events involved in tumor cell migration and metastasis,[33] demonstrating that a cell may utilize any number of means to achieve its end result and highlighting the fact that combined mechanisms may be employed which produce a phenotype which is resistant to attack on only one process.

***Cellular motility in invasive processes***

*Mechanisms of migration and invasion*

Tumor cell motility gains its first significance during tumor development, at the stage of in situ carcinoma. The most important factors at this stage are those which stimulate random kinesis or directed migration in an autocrine manner, thereby contributing to the disorganization of the tissue structure. This process is preceded or coupled to the acquisition of autocrine growth control. It is significant to note that numerous cytokines involved in autocrine growth control (eg. FGF, PDGF, IL-6) can also regulate cellular migration,[34-37] as detachment from the primary tumor mass is an essential aspect of tumor cell invasion. This process of detachment is determined in part by the loss of intercellular junctions during advancing grades of tumor dedifferentiation.[38,39] Early studies reported a decreased expression of desmosomal junctions in invasive carcinomas[39] and more recently it has been shown that downregulation or loss of the homophilic epithelial adhesion molecule E-cadherin or the E-cadherin-coupling molecule β-catenin suppresses the epithelial morphology and phenotype with a resulting switch to the expression of invasive fibroblastic characteristics.[40,41] Conversely, E-cadherin expression in fibroblasts suppresses migration as well as invasive behavior.[42] Changes in the expression of several other intercellular adhesion molecules of the immunoglobulin superfamily (i.e. NCAM, ICAM, CEA, DCC) are thought to be involved in the detachment of tumor cells as well, however their expression alone does not appear to correlate directly with tumor cell motility.[43]

Tumor cell detachment from the primary neoplasm is also determined by cell-ECM interactions, a primary mediator of tumor cell motility. Transition from in situ carcinoma to invasive carcinoma or from radial phase melanoma to vertical phase melanoma is characterized by transgression of the associated BM and, as such, discontinuous BM would appear to be required for the migration of single tumor cells or small aggregates into the adjacent connective tissue, however a clear correlation between

BM integrity and tumor malignancy cannot be established at this time.[39] Experimental as well as human tumor data have shown that intact basement membrane is frequently associated with high invasive and metastatic potential.[44,45] In an effort to determine a more useful prognostic indicator of invasive and metastatic capability, two migration-related processes have been proposed as diagnostic parameters: the presence of "tumor cell dissociation" at the invasion front (TCD),[46] and the presence of lymphatic vessel or blood vessel invasion by tumor cells (LVI, BVI).[47]

Basement membrane integrity in colorectal tumors is dependent on myofibroblasts at the tumor periphery and close opposition of tumor cells with these cells is required for synthesis of a typical basement membrane.[48,49] Collagen IV is synthesized by the myofibroblasts in this system, and disturbances in this cooperative endeavor result in the disorganization of the basement membrane, a phenomenon which can be exacerbated by heightened fibroblast motility in response to MSF, a molecule which is produced by the fibroblasts themselves presumably in response to tumor-secreted factors.[50]

In epithelial-derived tumor cells, contact with the collagen I matrix of the interstitium causes breakdowns in intercellular contacts without down-regulation of E-cadherin,[51] and similar disruptions have been observed in bladder carcinoma cells exposed to aFGF.[52] In contrast, HGF/SF, an important motility factor for epithelial tumor cells which is produced by fibroblasts in response to tumor secreted factors,[53] causes cellular dispersion by down regulating E-cadherin directly.[54]

Extracellular matrix-degrading enzymes such as MMP-2 and stromelysin-3, as well as uPA/plasmin are also produced by connective tissue cells in response to tumor interactions.[55-57] In addition to their enzymatic capabilities, proteases themselves have been reported to induce motility responses in tumor cells. For example, type IV collagenase and uPA were shown to be chemotactic for several different tumor cell lines,[58] and the receptor for uPA (uPAR) is localized in cells along the tumor periphery, while the enzyme itself is synthesized by adjacent fibroblastic cells.[55] This phenomenon of stromally-derived proteases interacting with tumor cell surface epitopes in a manner which enhances invasive characteristics appears to be related to enhanced tumor cell emigration from the peripheral edges of invading tumors and this proposal is supported by several lines of evidence including the observation that binding of exogenous uPA to uPAR induces the motility of epithelial cells[59] as well as the recently reported localization of MMP-2 to invading cellular processes via interaction with the integrin $\alpha v\beta 3$.[60] In this light it is interesting that a recent report described MMP-2-mediated detachment of cells from noncollagenous substrates.[61] Thus, collagenolytic enzymes such as MMP-2 may play important roles in multiple aspects of tumor cell invasion, both in mediating basement membrane dissolution as well as potentially facilitating the detachment of the tumor cell's trailing edge during migration. Indeed, integrin ligation events produce specific signaling events responsible for regulating metalloproteinase expression,[62,63] suggesting a reciprocal relationship involving integrins and MMPs which appears to involved in tissue remodeling events as well as invasive processes.

Integrins may therefore serve as more than mere mediators of cellular adhesion which provide the tractional forces requisite for cellular motility. Indeed, under certain conditions cells may require integrin-dependent signals which diagnose the extracellular environment and serve as anti-apoptotic mechanisms.[64,65] Although the role of cell adhesion molecules is of considerable importance in these processes of tumor progression, this topic is of too broad a scope for a review of this nature and is discussed in detail elsewhere.[43,66]

*Effects of matrix composition on migration and invasion*

In order to grow larger than 2 mm in diameter, tumors must enter an angiogenic stage in which they attract new blood vessel sprouts from existing vasculature to address the nutrient requirements of the growing neoplasm.[67] This event usually results in dramatic changes in the composition of the matrix surrounding the tumor, which in turn can affect tumor cell motility during invasive processes.[68,69] Angiogenesis is induced largely by peptide factors (eg. bFGF, VEGF, TGF-$\beta$, TNF-$\alpha$) produced directly by the tumor or via indirect mechanisms of intercellular activation in which connective tissue cells, responding to one or more tumor-associated stimuli, secrete angiogenic molecules and induce the infiltration of new blood vessels from pre-existing vascular circuits.[70] One of these molecules, vascular endothelial growth factor, was originally identified by its ability to heighten blood vessel permeability and was therefore designated vascular permeability factor (VEGF/VPF).[68] This molecule can have an important impact on the invasion of tumor cells located in the connective tissue since although this factor does not directly induce tumor cell motility, increased tumor-associated blood vessel permeability causes plasma components such as fibrinogen, plasminogen, fibronectin and vitronectin to cross into the organ parenchyma, thereby creating a chemo- or haptotactic gradient to which the tumor cell can now respond in an invasive fashion.

Deposition of a fibrin-containing matrix around the tumor is especially notable since, in addition to its direct promotion of tumor cell motility,[71] fibrin also enhances the angiogenic response which in turn increases the probability of vascular invasion by the cells of the neoplasm.[69] In addition, the endothelial cells which participate in the angiogenic process may contribute to the directed migra-

tion of tumor cells by releasing tumor cell migration-stimulating cytokines including IL-1 and IL-8,[72,73] as well as proteolytic cascade components such as uPA as well as type I and type IV collagenases,[74,75] and heparan sulfate-bound FGF complexes released by uPA-dependent proteolysis from endothelial basement membranes.[76]

As mentioned earlier, the density of ECM components regulates potential cellular interactions and an optimum matrix composition promotes tumor cell migration and invasion. How an intermediate ECM density is achieved in the peritumoral matrix is not clear, however several factors are known to contribute to this process. It is widely accepted that the majority of tumors lack lymphatic vessels but have extensive leaky blood vessels, thus contributing to the development of interstitial edema which acts to decrease the relative concentration of ECM components, and hence lower the resistance of the composite matrix.[38,39,68,69] Another mechanism for modulating the peritumoral matrix involves proteolytic enzymes produced by tumor cells as well as tumor-associated fibroblasts or endothelial cells. Proteolytic degradation of ECM components can both provide a conduit for invasion and decrease the relative density of the matrix, thus promoting enhanced chemo- or haptotactic movement of tumor cells in response to ECM degradation products.[55,56,74,75] Indeed, the complex regulation of motility by matrix composition and density is demonstrated by the fact that type I collagen degradation products are chemotactic for tumor cells,[77] while increased deposition of an intact type I collagen matrix results in desmoplasia, a condition which inhibits tumor invasion and metastasis.[78]

Several stromally-derived matrix components, including thrombospondin (TSP), tenascin (TN) and hyaluronic acid (HA) which are present in the peritumoral connective tissue appear to play a dual role with effects on both cell adhesion and motility.[79-81] TSP and TN have weak adhesive properties for cellular interaction, but more importantly are able to break existing cell-matrix adhesive interactions, limiting the adhesive strength of the matrix and favoring reduced spreading and/or cellular detachment.[82] In addition, TSP and HA stimulate tumor cell motility,[83,84] while it has been shown that a composite matrix of TN and fibronectin (FN) induces matrix metalloproteinase secretion by stromal cells,[63] thereby potentially indirectly regulating tumor cell motility by influencing both the presence of migration-modulating matrix degradation products and allowing tumor cell integrin- and non-integrin-dependent interactions with enzymatic components which are known to affect tumor cell motility as well.

Correspondingly, expression of specific integrins by the tumor cell may favor migration in the peritumoral matrix of origin, while non-integrin adhesion molecules may play a role in tumor cell migration in connective tissue as well. For example, CD44 and HRAMM may mediate tumor cell motility on HA,[83,85] a molecule which is increasingly deposited by fibroblasts in response to MSF.[81] In addition, tumor cells which express uPAR may have additional advantages over non-uPAR expressing cells during migration through vitronectin (VN)-containing matrices as receptor-bound uPA facilitates integrin $\alpha v\beta 5$-dependent migration in the absence of integrin $\alpha v\beta 3$,[86] and, since uPAR can also act as a receptor for VN.[87]

*Intravasation and extravasation of hematogeneous and lymphatic vessels*

Among the steps of the metastatic process, the molecular basis of intravasation is least understood, primarily owing to the lack of a suitable experimental model. Tumor cells must attach to the BM in order to initiate intravasation into the vasculature and numerous reports suggest that BMs are not symmetrical.[88-90] It is interesting to note, however, that the E8 fragment of laminin, which provides the binding site for the major laminin-binding integrins $\alpha 6\beta 1$ and $\alpha 7\beta 1$,[91] is predominantly oriented towards the lamina fibroreticularis although this fragment is also found in the upper part of the lamina rara, directly facing the cells.[88,89] In contrast, the central portion of the laminin molecule is localized to the border of lamina rara and lamina densa,[90] suggesting that this asymmetric localization of laminin in the BM may favor the attachment of tumor cell expressing $\alpha 6\beta 1$ and $\alpha 7\beta 1$ during the attachment phase of intravasation.

A peptide containing the IKVAV integrin-binding sequence of laminin's E8 fragment induced type IV collagenase activation in fibroblastoid tumor cells.[92] Similarly, migration through VN and type I collagen-containing matrices promotes expression of metalloproteinases as well, with the former interaction being mediated by the $\alpha v\beta 3$ integrin.[93,94] These phenomena may play an important role in successful attachment to and degradation of the vascular BMs encountered by the tumor cell. Indeed, structural changes in the walls of vessels located around or inside of tumors are common, and often involve the loss of BM electron density while BM is still detectable by immunohistochemistry.[95-97] Such changes in BM structure may involve proteolytic remodeling and could present a mechanism for both allowing passage of tumor cells through the BM barrier and potentially exposing cryptic adhesive sites, potentially further facilitating migration through the BM barrier. Indeed, specific cleavage of laminin-5 by MMP-2 induces migration of breast epithelial cells by exposing a cryptic promigratory site.[98] This altered form of laminin-5 is found in tumors and in tissues undergoing active remodeling, but not in quiescent tissues, highlighting the importance of this type of mechanism for invasive processes. Indeed, human melanoma cells require ligation of the $\alpha v\beta 3$ integrin to sustain viability in a dermal

collagen matrix, an event mediated by proteolytic exposure of a cryptic site within the type I collagen molecule.[65]

Penetration of the endothelium can occur intracellularly, intercellularly and by mechanical disruption of the endothelial lining.[97,99,100] In addition, special types of intravasation were observed including the case of a highly vascularized mammary tumor which involved lumenal entry of endothelium-covered tumor cell clumps, a process which probably does not involve motility of individual tumor cells.[101] Similarly, the entrance of the vasculature by large tumor cell groups which are continuous with the primary tumor mass, a process called permeation, appears to involve primarily sheet migration of large tumor cell groups, as was recently observed *in vitro* via coordinated modulation of cell-cell adhesion.[102,103]

The mechanism of cellular migration during unicellular intravasation is obscure because the leading edge is not attached to a substratum, and consequently, tractional force cannot be exerted through the lamella. In this case, the necessary force could potentially be provided by the cortical flow of actin, which involves local relaxation of the actin cortex where the leading edge is formed, accompanied by contraction of the opposite side of the cell.[7] This process does assume, however, that tumor cells are attached at their sides to endothelial cells. Another explanation relies upon tumor cell aggregates as described above, wherein the cell-cell interaction provides the required static force for the entering cells while the rearmost cells remain attached to the BM and/or stromal matrix adjacent to the BM.

In contrast, intravasation into lymphatic vessels is often presumed to occur at an early stage of tumor development, after the initial invasion of the epithelial BM and before the onset of angiogenesis,[38] however the presence of both experimental and human tumors which metastasize predominantly or exclusively through the lymphatic system suggests a regulated mechanism for this preferential mode of metastasis.[104-106] While there is currently no direct molecular mechanism which satisfactorily explains this metastatic phenotype, indirect arguments have been made based upon alternatively spliced variants of the HA-binding cell surface molecule CD44 of tumor cells and lymphocytes. The same variant [V6] which is transiently expressed on antigen-activated lymphocytes recirculating from the periphery of the body to the lymph nodes has also been observed on the surface of tumor cells which metastasize exclusively through the lymphatic system,[106-108] suggesting that this cell-matrix interaction imposes a predisposition for the lymphatic system upon the bearer cell. Overexpression of this variant also confers metastatic potential to nonmetastatic tumor cells,[109] however, it has been suggested that this splice variant may instead contribute to the subsequent growth of metastases in the lymph node, without direct effect on the processes of invasion and intravasation,[110] and at present, this possibility cannot be ruled out.

After entering the circulation, hematogenously metastasizing tumor cells are passively transported to the target organ. The entrapment and migration of tumor cells can be facilitated by nonspecific cytokines such as IL-1, IL-8, C3b,[72,73,94] as well as specific chemotactic factors originating from organ capillary endothelial cells,[111] a process exemplified by the identification of a lung microvessel endothelial cell-derived chemoattractant for lung-metastasizing large-cell lymphoma cells as monocyte chemotactic protein-1, a factor whose major function is the recruitment of monocytes to sites of inflammation which appears to facilitate site-specific metastasis of lymphoma cells to the lung.[112] Site-specific metastasis can be influenced by chemotactic factors secreted by endothelial cells. In addition, molecules derived from the ECM or stromal cells of the target organ itself which diffuse across the basement membrane from the organ parenchyma can also impact site-specific metastasis,[113-115] although these soluble factors are generally considered to have an affect on tumor cell motility only at the initial stages of extravasation, with later events mainly governed by the ECM of the target organ. Indeed, invading cells may metastasize via different routes and sequential failures in the dissemination process can contribute to the inability of individual tumor cells to establish final metastatic colonies.[116] Recent reports have further shown that a large proportion of intravenously-injected tumor cells are competent to extravasate, a step previously thought to present a great barrier to metastasis, and that subsequent growth after entering the new organ environment may instead provide the primary limitation to successful completion of the metastatic cascade once the cells have entered the bloodstream, further highlighting the multistage selective nature of these processes.[117]

Extravasation of tumor cells can take place via different mechanisms, including direct and simultaneous movement through both the endothelium and the BM,[118-120] delayed penetration through the BM, which involves temporary resting of the tumor cell on the BM while covered by endothelial cells (facing their basal side),[121] irreversible retraction of the endothelial cells followed by disruption of the BM by multiple cellular processes,[108,122,123] or intralumenal tumor cell growth and mechanical disruption of the vessel.[124] Furthermore, in larger vessels, extravasation may be preceded by the development of an intravascular tumor, which is covered by endothelial cells and faces the lumenal side of the BM.[121] It should be noted that the penetration of vessel walls during intravasation and extravasation need not take place by the same mechanism. For example, intravasation of tumor cells into lymphatic capillaries caused no damage to the endothelial cell lining, whereas extravasation of these same cells led to destruction of the lung capillaries.[108]

According to the previously suggested "docking and locking" hypothesis of cellular interaction with the endothelium, the processes of initial attachment of tumor cells to and subsequent rolling on the endothelium during cellular arrest after transport in the circulation are mediated by carbohydrate-carbohydrate (GM3-LacCer) and carbohydrate-protein (selectin) interactions, respectively, with subsequent binding stabilized by integrins.[85] The initial cell-endothelium interactions appear to be uninvolved in tumor cell migration through the endothelium as it was shown that PMN cells lacking CD18 are able to adhere to and roll on the endothelium but are not able to extravasate.[125] This phenomenon suggests a key role for integrins in migration through a vessel's endothelial lining, as is widely accepted for the role of integrin-mediated migration on the ECM, however it is not clear exactly how tractional force can be exerted on such a malleable surface as an endothelial cell. Integrin as well as non-integrin adhesion molecules on the surface of solid tumor cells can bind to the integrins present on the lumenal surface of the endothelial cells via bridging molecules such as fibrinogen or laminin[85] and direct binding to members of the immunoglobulin superfamily present on the surface of the endothelial cell is possible for integrins VLA-4 and LFA-1 the latter however, expressed on tumors of lymphoid origin. Recent results have shown another integrin, $\alpha v\beta 3$, is able to bind to the immunoglobulin superfamily member CD31,[126] as well as the cell-cell adhesion molecule L1.[127] These phenomena appear to play an important role in tumor cell migration through the endothelium since CD31 is localized predominantly to the interendothelial junctions[128] and L1 is expressed *de novo* on endothelium associated with disease states.[129] The binding between CD31 and v3 is significant as this interaction can lead to the dissolution of interendothelial junctions via substitution with tumor cell-endothelial cell contacts. Heterotypic binding has been observed between cell surface glycosaminoglycans and CD31,[130] however homotypic interactions may also be involved in cases of tumor cells expressing CD31.[131] As described above for intravasation, tractional force cannot be exerted through the leading edge during the process of extravasation, however it is possible that integrin-CD31 or integrin-L1 interactions at the tumor cell periphery could provide the scaffolding required for this force generation.

In contrast to intravasation, during extravasation tumor cells encounter vessels with intact BM. Consequently, although the chemotactic and haptotactic activities of the major BM components laminin and collagen IV are well documented, such activities during extravasation *in vivo* are questionable as these components are confined to a very thin (100-200 nm) layer of the BM,[132] and it is unlikely they would provide any sort of a gradient which could promote cellular migration under native conditions.

Penetration of the endothelium during extravasation without active cellular migration is also possible in cases where the tumor cell induces endothelial cell retraction. This process can be irreversible when mediated by $H_2O_2$ produced by invading tumor cells, and subsequent degradation of the subendothelial BM can take place during this process by proteolytic enzymes released by the damaged endothelial cells in addition to direct tumor cell action.[133] In contrast, reversible retraction events have been described as a result of endothelial cell exposure to a tumor-derived lipoxygenase metabolite of arachidonic acid, 12(S)-HETE.[134] This eicosanoid has been shown to upregulate $\alpha IIb\beta 3$ expression on melanoma cells as well as $\alpha v\beta 3$ expression on the lumenal surface of endothelial cells, thereby promoting stronger binding between the tumor cells and the endothelium by enhancing bridging effects as described above.[85] Endothelial monolayer retraction can also be caused by interactions with ECM components such as fibrin, VN and FN can also contribute to this process.[135] Indeed, when placed on endothelial monolayers, fibrin-covered microspheres cause monolayer reorganization in such a way that the microspheres are relocated to the basal side of the endothelial layer. *In vivo* a similar process can mediate the removal of the tumor cells and tumor cell-platelet aggregates which display particular ECM molecules on their surface from the circulation, followed by restoration of an intact capillary wall.

Subsequent to the traversing of the BM, tumor cell migration is largely influenced by the connective tissue of the target organ,[113,114,136] or by cell-cell interactions within this new environment.[137] The stromal face of the target organ BM also serves as a suitable surface for tumor cell migration, however, as has been observed for both endothelial or epithelial BM's *in vitro* and *in vivo*.[138,139] In accordance with the previously described asymmetry in BM structure, migratory interactions with the alternate sides of the BM may involve a different set of ECM receptors. For example, the binding site for the $\alpha 2\beta 1$ integrin and, for the 67 kDa laminin/elastin binding protein is located on the $\beta 1$ short arm of laminin,[140,141] while a different binding site for the latter has also been delineated on the long arm of laminin near the intersection of the arms.[142] Since the central portion of the laminin molecule is located at the border of lamina rara and lamina densa in the BM, these adhesion molecules probably mediate migration on the cellular side of the BM, while the $\alpha 6\beta 1$ integrin may be involved, in tumor cell migration on either side as described above. It should be noted that the organs most frequently targeted by the metastatic process (liver, lung, adrenals, brain) have a very low connective tissue:parenchyma ratio, which limits the space for migration after extravasation and restricts the types of adhesion receptors which can be

employed as well as the spectrum of growth factors/cytokines which might play a role in further phases of tumor cell growth in this new organ environment.

### *Soluble factors and cellular locomotion*

#### *Mitogenic versus motogenic responses*

At different times during the metastatic process, tumor cells bifurcate between proliferative processes and migration/invasion responses to various soluble factors in the extracellular millieu. The differential tendency in favor of one response or the other can be regulated at several levels, starting with the soluble cytokine itself. In the primary neoplasm the differentiation grade or dispersion state of the tumor may be directly responsible for such discrete responses. It has been reported recently that FGF or EGF can promote distinct proliferation or motility responses in bladder carcinoma cells dependent on their culture density such that subconfluent cultures responded with src kinase-dependent scattering and motility, whereas confluent cultures exhibited proliferation.[143] In addition, the available concentration of soluble factors can dictate differential cellular responses including motogenic or mitogenic stimulation. For example, PDGF-BB has been shown to activate different signaling pathways which result in motile responses associated with elevated FAK phosphorylation at low concentrations, in contrast to proliferative responses at higher concentrations.[144] Another example of concentration-dependent regulation of differential motogenic or mitogenic responses is provided by RANTES, a chemokine for memory T cells, which at low concentrations causes transient G protein-mediated increases in intracellular calcium which result in migration, while at high concentrations, this cytokine produces a tyrosine kinase-dependent sustained influx of calcium which drives the cell into a proliferative response.[145] Differential responses can also be initiated by the generation of multiple cytokine isoforms via alternative splicing. For example, the mesenchymal-derived cytokine hepatocyte growth factor was originally described as scatter factor due to its chemokinetic effect on cultured epithelial cells, and is now referred to as HGF/SF.[35] This molecule is processed into multiple splice variants and it has been demonstrated that the smaller species is sufficient to transduce the motility signal of HGF/SF, whereas the whole molecule is necessary for the induction of mitogenesis.[146]

Another level at which regulation of migratory versus proliferative responses can be modulated involves the qualitative or quantitative expression of cellular receptors. In the case of the insulin-like growth factors (IGF's), the IGF-II receptor was shown to mediate motility of rhabdomyosarcoma cells, for which this cytokine is an autocrine regulator of motility, while the IGF-I receptor was responsible for transmitting mitogenic signals to these cells.[147] Similarly, in endothelial cells the PDGFβ receptor is coupled with a motility response whereas the PDGFα receptor is unable to transduce such a signal.[148] In contrast, integrins can transmit mitogenic signals as well,[149] and laminin adhesion events stimulate melanoma cell proliferation in a manner which is dependent upon the cooperative interaction of α3β1 and α6β1 integrins.[150] In this case, integrin α3β1 was shown to play an inhibitory role during migration of these cells, suggesting that ECM components may induce cellular proliferation or migration responses which are at least in part dictated by the integrin profile of the cell. Indeed, although these processes may appear distinct, complex interdependencies have been observed during execution of more elaborate functions. The IGF-1 receptor, long known to function as a motility receptor,[37] cooperates with the αvβ5 integrin to promote tumor cell dissemination and successful completion of the metastatic cascade by otherwise metastatically incompetent cells.[151]

The final bifurcation of the mitogenic or motogenic response of a cell to a given cytokine may be invoked at the level of receptor-coupled signal transduction pathways. In the case of the EGF receptor, kinase activity is sufficient to induce proliferation alone, however autophosphorylation was necessary for a motility response.[152] The importance of autophosphorylation in stimulating motility was also demonstrated for the PDGF and HGF/SF receptors and it is required for the recruitment of SH2 domain-containing effector molecules which are involved in mitogenesis (eg. PLCγ, PI-3K, rasGAP).[153-155] The HGF/SF receptor has a multifunctional docking site for PLCγ, PI-3 kinase (PI-3K), GRB2 and/or src kinase, and it has been proposed that distinct phosphorylation profiles of the receptor may regulate which effector protein will be coupled.[156] The EGF receptor, on the other hand, interacts specifically with PLCγ which mediates rearrangement of the actin cytoskeleton.[157] In contract, the PI-3K pathway appears to specifically transduce motility signals from the PDGF and HGF/SF receptors since deletion of the PDGFβ receptor domain which is responsible for binding of PI-3K abolished PDGF-induced motility in the absence of proliferation.[158] In addition, inhibition of PI-3 kinase negatively influenced motility signaling through the HGF/SF receptor,[159] further implicating receptor-mediated signal transduction as a critical mediator of soluble factor effects.

Receptor phosphorylation represents a means of clustering effector molecules in specific spatial relationships requisite for the induction of ligand-induced signaling processes. For example, autophosphorylation of the receptors for FGF and insulin appears to be necessary for motility induction, and in both cases this migratory response was dependent on pertussis toxin-sensitive G-proteins,

however these receptor-coupled pertussis toxin-sensitive G protein were not required for proliferative responses through these receptors.[160,161] Similarly, transphosphorylation events can differentially regulate motility and proliferation of cytokine-treated target cells as well. For example, whereas PKC inhibition enhanced the proliferative response mediated by the c-kit/CSF receptor, PKC was absolutely required for motility induction by CSF.[162]

*Specific soluble mediators of cellular migration*

As can be surmised from the preceding sections, the processes of tumor metastasis in general and cellular migration in particular are complex phenomena which require the concerted effort of numerous effectors. Accordingly, the role of a single component must be assessed with regard to the significance of coordinating molecules. As noted above, soluble factors can have dramatic effects on a cell's motile properties, however the potential regulation of migration to the exclusion of growth effects in recipient cells suggested to researchers the presence of motility factors, a group of molecules whose primary influence would be locomotory in scope. As such, investigations were undertaken to prove or disprove the "motility factor" theory by identifying molecules which solely impacted cell migration.

These factors fall into two broad categories, paracrine and autocrine mediators. Paracrine factors are proposed as potential mediators of site-specific invasion and metastasis events, and are generally produced by stromal cells of the host. The prototypical paracrine molecule of this class is Scatter Factor, originally defined as a fibroblast-derived inducer of epithelial loss of cell-cell adherence which has since been identified as hepatocyte growth factor (HGF/SF).[35] More recent additions to this class of effectors are Epitaxin, a fibroblast-derived stimulator with effect on a broad spectrum of epithelial tumor cell types,[163] and a group of tumor or peritumoral fibroblast-derived motility-stimulating factors (FMSF's) which enhanced the migration and of sarcoma cells selected from lung-metastases.[164] Other paracrine mediators have been described to various degrees, however the potential appeal of these factors is obvious in terms of specificity of response: if a competent cell encounters the factor, it can respond to it. What happens when the competent cell produces the very molecule it wishes to respond to?

Various reports have demonstrated the production of autocrine factors which, by their very nature, act upon the cells which secrete them. Notable among these is a migration-stimulating factor (MSF) distinguished during analysis of the differential effect of cell-density on migration of adult versus fetal or breast cancer-derived fibroblasts into a three-dimensional collagen matrix which resulted from differential production of this factor.[50] This effect turned out to be due to enhanced production of hyaluronic acid (HA) in response to MSF,[81] a process which is antagonized by TGF-1.[165] More recent autocrine factors of note include a glioma-produced motility factor (GMF) implicated in invasion of this tumor type,[166] as well as an invasion-stimulating factor (ISF) produced specifically by metastatic prostate tumor variants,[167] which appears to mediate invasive characteristics in part by up regulating MMP-2 secretion.[168] Interestingly, two distinct factors have been described recently from highly metastatic pancreatic cancer cells, a dissociation factor (DF)[169] and a pancreatic-derived motility factor (PDMF),[170] both of which were distinguished by their ability to induce effects in weakly metastatic variants of the producing tumor cells, thus defining a potentially novel autocrine/paracrine effector pathway of significance in the progression of pancreatic cancer. The production of autocrine factors is not restricted to transformed or embryonic cells, as normal smooth muscle cells (SMC's) secrete a potent SMC-derived migration factor (SDMF) implicated in locomotion of these cells within vascular walls and in the intimal thickening associated with atheroma formation.[171]

Other factors of each group have indeed been described, and several outstanding reviews on the role of these molecules as well as growth factors in cell movement are available.[35,172,173] It is interesting to note certain common themes among these motility factors, including the proteinaceous nature of the molecules themselves, most exhibiting significantly larger molecular weights than is characteristic of the classic polypeptide growth factors, as well as their lack of effect on target cell proliferation, whether their mechanism is autocrine or paracrine. The finding of this diverse group of molecules arising from such varied cell types highlights the numerous regulatory steps important to such a complex process as cellular migration.

## ***Tumor cell autocrine motility factor and its receptor***

*History of AMF*

AMF was identified by Liotta and colleagues in 1986,[174] as a proteinaceous molecule produced by human melanoma cells which stimulated the locomotion of the very cells which produced it, thereby providing its name. Since AMF was also produced by ras-transformed 3T3 fibroblasts but not their untransformed kin, AMF appeared to have all the hallmarks of a tumor cell-specific motility factor with potential relevance to invasion and metastasis. Interestingly, the locomotory impetus of this molecule appeared to be at least as much chemokinetic as chemotactic, suggesting that AMF might merely provide the impetus while the directionality of the locomotory response might be determined based upon unrelated information about the cellular microenvironment. Further

investigations into AMF activities the following year described the purification of AMF from a human breast carcinoma cell line suggested that the induction of pseudopodial protrusion is a requisite event in the cellular locomotory response to AMF.[175] Attempts to delineate AMF intracellular signaling events soon revealed that a *Bordella pertussis* toxin (PT)-sensitive G-protein,[176] as well as inositol phosphate metabolism mediates cellular responses to AMF stimulation,[177] however much remained to be determined regarding AMF's mechanism of action. For instance, was its effect due to a specific receptor-mediated pathway? Were there differences in phenotypic responses of high- or low-metastatic cells? Does AMF play a role in tumor progression and metastasis?

The answer to the first question came indirectly as a result of efforts to determine cell surface molecules which displayed differential glycosylation in murine melanoma cells selected for reversibly enhanced metastatic capacity.[178] One such molecule, a sialylated 78,000 Da integral membrane protein (termed gp78/AMF-R) was selected and a monoclonal antibody was raised against it. Treatment of living cells with this antibody stimulated their migration, while pretreatment with Fab' fragments promoted lung colonization in an experimental metastasis assay, suggesting that gp78 might play a role in cellular locomotion during metastatic processes.[179] In addition, concentrated tumor-conditioned medium from these melanoma cells also contained a component which enhanced migration of the producing cell population (similar to AMF, which had only recently been described), and locomotory responses to both the tumor-conditioned medium and the anti-gp78 antibody were abolished by pretreatment of the cells with PT at concentrations which suppressed AMF-induced migration, suggesting that gp78 might be mediating AMF responses. Further identification of gp78 as the receptor for AMF came when pretreatment of cell lysate immunoblots with tumor-conditioned medium inhibited binding of the anti-gp78 mAb to its antigen.

Having established a potential receptor for a crude motility factor preparation, Raz and colleagues went on to purify AMF and gp78 from the murine melanoma in which gp78 was originally identified, and they used these materials to establish that AMF does indeed bind to gp78 and stimulate motility in a dose-dependent manner.[180] In addition, AMF was purified and gp78 was cloned from a human fibrosarcoma cell line.[181] These preparations of AMF appeared to be homologous molecules, exhibiting single polypeptide chains with similar molecular weights of 64 KDa under reducing conditions and 55 KDa under nonreducing conditions, similar to those reported previously and indicating the presence of intrachain disulfide bonds.[174] In addition, each protein displayed multiple species under isoelectric focusing, with acidic pI's between 6.1 and 6.4. The sequence of gp78 showed characteristics typical of a transmembrane molecule (eg. leader sequence, transmembrane domain, extracellular N- and O'-linked glycosylation motifs) as well as certain signatures of a ligand-induced receptor as well, among these a nucleotide-binding domain typical of serine/threonine kinases, as well as an intracellular consensus site for protein kinase C phosphorylation, suggesting potential cross-talk of signaling molecules during cellular responses to AMF.

Elucidating the molecular identity of the AMF ligand itself proved to be somewhat more troublesome than the receptor, gp78, however studies into the mechanism of AMF action and the expression of its receptor continued. Initial attempts to characterize the gp78-mediated signaling events coupled to the AMF locomotory response determined that gp78 becomes phosphorylated in response to cytokine treatment[181] and later studies demonstrated that phosphorylation occurred on one or more serine residues (and potentially tyrosine as well) (S. Silletti, unpublished observation) The use of enhanced kinesis as a phenotypic readout and receptor phosphorylation as a biochemical readout of AMF activity now allowed more detailed analyses of AMF-mediated signaling processes to be done.

*Mechanistic translation of AMF stimulation into a motility response*

During the original description of AMF's effects it was noted that quinacrine, an inhibitor of phospholipase A2, markedly reduced migratory responses to AMF.[182] Furthermore, membrane phospholipid methylation and locomotory responses to AMF were both suppressed by the methylation-inhibitor deaza-adenosine, whereas AMF treatment in the absence of this compound reciprocally promoted a sustained increase in the methylation of phosphatidylcholine, the major substrate for phospholipase A2, further suggesting a role for arachidonic acid metabolism in AMF-induced responses. Accordingly, more recent investigations showed that perturbation of gp78 directly stimulates a PT-sensitive G-protein mediated increase in 12-lipoxygenase (12-LOX) activity and expression in high- but not low-metastatic melanoma cells which yields elevated levels of the eicosanoid 12(S)-hydroxyeicosatetraenoic acid [12(S)-HETE], a LOX metabolite known to alter cytoskeletal architecture in melanoma cells.[183] Reciprocally, exogenously-added 12(S)-HETE stimulated migration of the highly metastatic cells only, with concomitant phosphorylation of the AMF-R and upregulation of surface AMF-R levels and reorganization of this receptor to the cellular protrusions and cell edges in a PKC-dependent manner. These components of the "autocrine motility cycle" (i.e. gp78, 12-LOX and PKC) have been shown to be overexpressed by highly aggressive but not less aggressive human prostate carcinoma cells *in vivo*,[184] further implicating this system in tumor malignancy.

The high-affinity αIIbβ3 integrin has recently been shown to be involved in the invasion of human melanoma cells *in vitro*,[185] a phenomenon which is PKC-dependent and can be stimulated specifically with the LOX metabolite 12(S)-HETE, a known second messenger of the AMF/gp78 pathway. Analogously, previous studies showed that AMF-R occupancy increased melanoma cell adhesion and spreading on fibronectin via upregulation of surface αIIbβ3 and α5β1 integrin receptors.[186] This AMF-induced translocation of integrins from the cytoplasm to the cell surface occurred in disparate ways, however, with preferential redistribution of αIIbβ3 from the apical cell surface to the cell periphery without effect on the localization of α5β1 in a PKC- and 12(S)-HETE-dependent manner. Furthermore, AMF-enhanced invasion of a reconstituted basement membrane barrier by these melanoma cells was susceptible to inhibition by antagonists of integrin αIIbβ3 but not α5β1, further delineating the role of integrin αIIbβ3 in AMF-mediated melanoma cell invasive processes.

A potential role for the cysteine protease cathepsin B in AMF-stimulated motility was originally suggested when inhibitors of this enzyme suppressed AMF-induced motility of melanoma and carcinoma cells under assay conditions which did not present the cells with a physiological barrier requiring enzymatic degradation.[182] In addition, a cysteine protease inhibitor suppressed AMF-induced locomotory induction as well as *in vitro* invasion and *in vivo* metastasis of bladder cancer cells.[187] Interestingly, cathepsin B is located primarily in a cell membrane-associated form in highly metastatic melanoma cells, whereas weakly metastatic variants maintain a cytosolic localization.[188] In addition, the AMF second messenger 12(S)-HETE stimulates the release of this enzyme from malignant cells via a PKC-dependent mechanism.[189] Several possibilities exist which could explain the requirement for this enzyme in AMF-mediated migration, including the possibility that AMF directly stimulates cathepsin B activity within the membrane to promote cleavage of another proenzyme from its latent to active form, the active form of which may be specifically required for the AMF motile response, as described previously.[190] More likely, a situation analogous to that described above for uPA/uPAR[86] may be occurring, as cysteine protease inhibitors have also been shown to inhibit migration on (and to a lesser extent adhesion to) ECM proteins,[191] suggesting the effect of cathepsin B on AMF-stimulated motility is probably indirect rather than direct.

*AMF responses are related to a cell's intrinsic phenotype*

Phenotypically, AMF was originally purported to constitute a tumor cell-specific effector, however the altered glycosylation of its receptor in response to growth conditions which favored enhanced metastatic capacity suggested that AMF might preferentially affect high-metastatic cells over their low-metastatic counterparts. In contrast to the enhanced migration evinced by highly metastatic murine melanoma variants, low-metastatic clones proved to be generally refractory to locomotory stimulation via gp78 perturbation, an effect which translated into an absence of increased lung colonization in the low-metastatic variants, while marked enhancement of pulmonary metastasis was noted in the high-metastatic variants.[192] This differential effect is in line with the differential intracellular signaling events noted above for high- versus low-metastatic melanoma cells, and is related to the differential surface localization of gp78 on these cell types. Although the high-metastatic cells display polarized gp78 primarily at their leading edge, the low-metastatic cells demonstrate multiple punctate regions of gp78 around their entire periphery. Furthermore, priming of the high-metastatic cells by preincubation with PT prevented internalization of gp78-antibody complexes resulted in a marked increase in pulmonary metastasis which was directly related to decreased surface expression of gp78 following washout of the PT prior to injection. These findings suggested that directed endocytosis to form a single leading edge is related to the intrinsic metastatic ability of these melanoma cells and that perturbation of gp78 through receptor occupancy promotes cellular kinesis during metastasis through receptor-ligand complex internalization. Similar differential effects of soluble factors dependent upon the intrinsic phenotype of the recipient cell have been described previously, including opposing effects of soluble fibroblast-derived mediators on the growth of radial versus vertical growth phase cutaneous melanoma cells[193] and contrasting responses to TGF-β in metastatic versus primary prostate tumor cells.[194]

Although immortalized 3T3-A31 fibroblasts do not secrete AMF, they do however express AMF receptor[195] and are capable of responding to the locomotory stimulus of this cytokine.[196] In fact, these cells exhibit a dose-dependent proliferative response to AMF in addition to the kinetic effects of this cytokine. These findings raise questions as to whether AMF is truly an autocrine factor whose primary effect is on migration, and whether those effects are restricted to tumor cells. To address this concern, sublines of the 3T3-A31 fibroblast which exhibit transformed but not tumorigenic, tumorigenic but not metastatic, and fully metastatic phenotypes were utilized for comparison with the parental line to demonstrate that cell-cell contact downregulates AMF-R expression in the parental cells, whereas this phenomenon is progressively lost in concert with advancing stages of dedifferentiation.[197] Similarly, although the parental 3T3-A31 cells display a narrow window of proliferative response to AMF, the bimodal loss of mitogenic response to AMF at higher concentrations in these untransformed parental cells is progressively lost as a function of phenotypic progression as well.

The decreased expression of AMF-R at high cell density observed in the parental 3T3-A31 cells is due to diminished promoter activity as a result of interaction with specific DNA-binding proteins which are differentially expressed by dense and sparse fibroblast cultures.[198] Importantly, HeLa cervical carcinoma cells displayed gel shift patterns which paralleled those of sparsely-cultured 3T3 fibroblasts irrespective of culture density, suggesting that direct or indirect loss of promoter responsiveness to DNA-binding proteins or an altered expression profile of transcription factors at high cell density facilitates the inappropriate continued expression of gp78 in high cell-cell contact conditions.

That this continued AMF-R expression alone is not sufficient for the metastatic phenotype is evidenced by the fact that the transformed and tumorigenic 3T3-A31 variants exhibit persistent AMF-R expression at high cell density but do not display heightened migration in response to AMF, whereas the parental and metastatic cells are capable of responding to AMF's locomotory influence. This discrepancy is explained by the differences in localization of AMF-R on the cell surfaces of these variants. The migrationally-inducible cells display monofocal staining for gp78, reminiscent of highly-metastatic melanoma cells which are also capable of AMF locomotory responses, while the uninducible variants evince gp78 clusters randomly distributed around the cell periphery, representing the counter-productive extension sites of multiple leading edges as described previously for low-metastatic melanoma cells.[195,199] In addition, AMF promoted marked rearrangement of focal adhesion plaque proteins and tyrosine phosphorylated proteins in AMF locomotory-responsive cells exclusively, an effect which appeared to be independent of changes in the phosphorylation state of pp125FAK.[197,200] This data is in line with the facts that the AMF response proceeds through the serine/threonine kinase PKC,[183,184] and that the AMF receptor contains a characteristic serine/threonine kinase motif and is phosphorylated on serine.[181] Therefore, the stimulation of cellular migration by AMF may be independent of tyrosine phosphorylation events at the focal contacts and may therefore represent a novel pathway of cytokine-induced motility regulation.

*The AMF system as a marker of malignancy*

Differential responses between tumor cells of differing metastatic phenotype as well as the capacity for inappropriately continued expression of AMF-R in high cell density growth conditions suggests that AMF may play a role in tumor invasion and metastasis *in vivo* and that expression of its components might serve as a relevant prognostic indicator of tumor malignancy. Early reports of AMF-like activity as well as the presence of detectable AMF protein in urinary voids of patients with cancers of the urinary tract and bladder demonstrated that AMF levels in these specimens correlated well with other more established methods of prognostic determination,[201,202] providing a potentially noninvasive screening modality similar to that described previously for bFGF levels in the urin.[203] More recently the receptor for AMF, gp78, was suggested as a potential urine marker for transitional cell carcinoma of the bladder,[204] indicating that the AMF system might serve as a prognostic indicator at least in the uro-genital system.

*In vitro* analysis of cell lines derived from human normal, noninvasive papilloma and transitional cell carcinoma urinary bladder tissue demonstrated that whereas the papilloma cells were immotile, the normal and carcinoma cells exhibited similar basal migration, however only the carcinoma cells were capable of responding to tumor-derived AMF with a locomotory response.[205] Cell surface distribution of AMF-R distinguished the three populations, with localization of gp78 to a single leading edge in the migrationally-competent carcinoma cells exclusively. Importantly, while cell contact downregulated AMF-R expression in the normal but not the carcinoma cells, AMF-R expression in the immotile papilloma cells, which grew as large cellular aggregates under high cell density conditions, was restricted to the external edges of the peripheral cells, possibly indicative of an intermediary stage of dedifferentiation in the disregulation of the AMF pathway.

It is important to note, however, that differences in migratory phenotype and gp78 expression profiles in these cells could not be correlated to changes in gene structure or copy number, indicating that the differences observed in AMF-R regulation are probably due to indirect effects of alterations in other regulatory components such as the DNA-binding protein profile expressed by malignant cells under conditions which would render their normal counterparts quiescent (eg. high cell density for these epithelial-derived urinary bladder cells). That the AMF system may play a causal role in the invasive stages of bladder cancer is supported by the finding that inhibition of PKC suppresses invasion characteristics of human urinary bladder carcinoma cells,[206] consistent with AMF-mediated intracellular signaling.

Indeed, immunohistochemical examination of AMF-R expression in specimens from human bladder carcinoma patients revealed a strict negative correlation between AMF-R expression and prognosis.[207] In fact, while normal urothelium does not express AMF-R, high levels of the intracellular homotypic cell-cell adhesion molecule E-cadherin are normally observed. Importantly, the increased AMF-R levels observed in the more severe bladder carcinomas described in this report (eg. invasive and metastatic as well as superficial which later progressed to advanced disease) were associated with a concomitant loss

of E-cadherin expression. This data suggests that enhanced migration of bladder carcinoma cells associated with invasion of the suburothelial lining is due to both the acquisition of motility-promoting molecules such as gp78 as well as the loss of molecules which promote the sedentary phenotype (eg. E-cadherin) and indicates the potential utility of a dual-antigen approach for improving early diagnosis of high risk bladder cancer patients, especially in the cases where the normal predictors of stage and grading fail (as in superficial, localized lesions which progress in a small proportion of cases and cannot be predicted by current methods). Interestingly, while the *in vitro* bladder papilloma aggregates described above exhibit E-cadherin expression which is restricted to cell-cell junctions, gp78 expression was limited to the external edges of peripheral cells, representing mutually exclusive subcellular distributions which suggests that peripheral cells which develop decreased E-cadherin levels would likely be driven to dissociate from the primary tumor mass due to a heightened migratory phenotype, providing a possible means for progression from benign noninvasive papilloma to potentially invasive bladder carcinoma.

Employing the well-characterized MDCK *in vitro* model system of polarized epithelial cells, these findings have been further elaborated to show that the normally minimally motile MDCK epithelial cell phenotype is predominated by high levels of E-cadherin and low-levels of gp78, while Maloney sarcoma virus-transformed MDCK variants which exhibit progressively higher intrinsic migration rates display correspondingly diminished levels of E-cadherin coupled with a steady-state high level of AMF-R..[208] These data confirm that loss of the polarized phenotype and increased cellular migration after transformation of these epithelial cells is associated with a shift from a high E-cadherin/low AMF-R profile to one of low E-cadherin/high AMF-R during the transition from a sedentary to a migratory phenotype. Interestingly, the gene for gp78/AMF-R was mapped to the long arm of chromosome 16, band q21 just distal to 16q13, a region of ample interest in relation to common epithelial tumors as well as leukemias.[205] Of special interest is the fact that the gene for E-cadherin is located at 16q22.1,[209] although the significance of the proximity to the gp78 gene locus is unknown. It is reasonable to speculate, however, that chromosome 16 alterations might result in the coordinated disregulated expression of both proteins to yield the patterns observed above, thereby promoting loss of the polarized epithelial phenotype and heightened cellular motility, culminating in tumor invasion.

The possible utility of members of the AMF system as prognostic indicators in other systems was suggested by the finding that oral squamous cell carcinoma cells produce an AMF-like molecule,[210] and an AMF-like substance from a primary epidermoid carcinoma of the oral cavity has been described as well.[211] Indeed, direct immunohistochemical examination of tissue from esophageal squamous cell carcinoma patients demonstrated that AMF-R expression was significantly associated with tumor size, infiltrative growth, depth of invasion and lymph node metastasis.[212] Moreover, the cumulative survival rate of patients whose tumors expressed gp78 was significantly lower than that of patients whose tumors were gp78-negative. These findings implicated the AMF system in progression of cancers of the gastrointestinal tract and suggested that quantification of gp78 levels in other tumors of this system might serve as a useful prognostic indicator of malignancy.

Further analysis using gastric cancer specimens demonstrated that expression of gp78 was associated with macroscopic type, lymphatic and venous invasions, and lymph node and peritoneal metastasis.[213] Importantly, gp78 expression correlated with clinically accepted parameters including histological grade and stage, and positive gp78 expression was significantly associated with poor prognosis, suggesting the potential utility of gp78 as a prognostic marker in this system as well. In the case of colorectal cancer, AMF-R expression appears to be especially informative as gp78-positivity correlated with tumor progression as reflected by histologic type, depth of invasion, lymph node metastasis, vessel invasion and tumor stage and predicted a poorer survival rate as well as higher incidence of disease recurrence in individuals who underwent curative resection.[214] Taken together, these results indicate that the AMF system may be a prominent player in the progression of tumors arising in the gastrointestinal tract.

More recent studies have determined that AMF-R may play a role in choriocarcinoma[215] as well as cutaneous malignant melanoma as well.[216] This latter finding was predicted by both the original characterization of AMF and gp78/AMF-R from cutaneous melanoma cells and the differential gp78-mediated effects in melanoma cells of differing metastatic capability. Indeed, the previous *in vitro* characterization of differential gp78 expression and responsiveness to AMF locomotory stimulation of human breast[200] and prostate cancer cells[217] suggests the potential utility of these molecules in other systems as well, significantly in the realm of hormone-responsive tumor types.

A discernible extension of AMF's potentially general role in invasive processes has come in the form of a report which describes AMF-like activity in the synovial fluid of rheumatoid arthritis patients.[218] Rheumatoid arthritis shares many features with neoplastic disease and is characterized by cytokine-dependent events which are analogous to tumor progression, including extensive neovascularization and invasion/erosion of synovium-lined joint structures including the patella of the knee.[219] This report of AMF-like activity in rheumatoid synovial fluid suggests that the AMF system may be a common functional component of

processes involving invasive cellular characteristics and indicates that this disease might be susceptible to treatments aimed specifically at disrupting the AMF system.

*Autocrine motility factor as an example of the emerging class of ecto/exoenzyme cytokines.*

Until recently, the molecular identity of AMF had proved extremely elusive. Despite reports of N-terminal sequences during the early days of AMF characterization[175,220] and the generation of antibodies against this molecule,[201,202] final identification of AMF determined that the N-terminus was blocked, similar to original descriptions of the molecules sharing identity with AMF.[221,222] Internal sequence analysis of AMF did, however, reveal an identity which was borne out by further biochemical and immunological analysis. The AMF protein has now been identified as a molecule previously designated neuroleukin (NLK), a secreted cytokine identical to the glycolytic enzyme phosphohexose isomerase (PHI) which catalyzes the reversible isomerization of glucose-6-phosphate to fructose-6-phosphate.[223] This finding is somewhat surprising in that this is a cytosolic enzyme without an apparent secretory signal, however prior reports have characterized soluble NLK as well as PHI which was not cell-associated, suggesting that this enzyme can indeed be released from the intracellular compartment.[224-226]

PHI/NLK is a polypeptide comprised of 558 amino acids with a predicted Mr of 63,189 Da (human) and 62,803 DA (mouse) and, although the ORF of PHI/NLK contains 3 potential N-linked glycosylation consensus sequences, no sugar modifications have been identified in these molecules,[222] analogous to findings with AMF.[180] Paradoxically, whereas the isoelectric points of human and murine AMFs were identified as acidic, within a nominal range of 6.1 to 6.5,[180,181,227] the reported pI of human PHI is 9.2.[228] The reason for this apparent discrepancy is unknown, however multiple isoelectric species were demonstrated for murine and human AMF's, and three PHI variants were described from a human gastrointestinal carcinoma with pI's of 9.1, 8.9 and 8.6,[229] suggesting some variability in composition from source to source. Indeed, multiple amino acid point mutation substitutions have been identified in PHI from patients presenting with a rare form of nonspherocytic hemolytic anemia,[230-233] and mutations in glycolysis deficient cells have also been described,[234] therefore it is not unlikely that variants of this molecule are produced by cancerous cells, potentially explaining this discrepancy in isoelectric point. In addition, it is possible that posttranslational modifications such as amino acid modification (which could explain the N-terminal blocking of the -amino group of these proteins), phosphorylation or potentially glycosylation may be responsible for these differences in observed pI.

The possible presence of mutant forms of this polypeptide has been difficult to validate as only one chromosomal location has been delineated for PHI and NLK, that being the long arm of chromosome 19, and restriction mapping of human DNA suggests a single gene while no evidence has been found for more than a single species of mRNA.[222,225] Nonetheless, it is significant that cases have been described in which physiological distinct phenotypes result from alterations in the NLK/PHI primary protein sequence. In addition to the hemolytic anemia and glycolytic deficit conditions described above, a conserved sequence of the gp120 protein of the HIV virus-1, or a synthetic polypeptide based on this sequence, which exhibits sufficient conformational similarity to NLK to bind NLK's effector molecule on neuronal cell surfaces, thereby causes or contributes to the development of AIDS-related dimentia, an indirect and previously unexplainable result of HIV-1 infection.[235,236] These findings suggest that distortion of PHI/NLK conformation by subtle sequence variations (as found in hemolytic anemia and potentially in cancer) may contribute to the different physiological effects of these molecules (NLK or AMF) on recipient cells.

Another enigma arises during comparison of the conformational requirements of each aspect of the pleuripotent effects of a single molecule with multiple identities such as this. The predicted peptide sequences of human and mouse forms of NLK and PHI contain 4 cysteines which are available for disulfide bonding, therefore this could readily explain the different relative migrations observed for AMF and NLK under reducing and nonreducing conditions.[223] However, it is interesting to note that although reduction of the disulfide bonds of AMF abolishes AMF's ability to stimulate cellular migration,[174] these bonds are not required for the glycolytic enzymatic activity of pig PHI, a molecule which contains only three of the four cysteines present in the mouse and human forms.[221] Similarly, it has been suggested that mouse NLK does not require disulfide bonding for its function, although the data responsible for such a claim are muddled at best, and mouse NLK displays a nonreduced apparent molecular weight of 56 KDa, suggesting the presence of disulfide bridges in its native conformation.

NLK was identified as a trophic factor which promotes the *in vitro* survival of a subpopulation of spinal skeletal motor neurons as well as cultured sensory neurons which are insensitive to nerve growth factor (NGF), without apparent effect on sympathetic or parasympathetic neurons.[222] Tissue distribution of NLK demonstrates relative expression levels of skeletal muscle>brain>>heart, kidney, testes>liver and salivary gland, however low levels of NLK were observed in serum, lung, thymus, spleen, ovary, adrenals and pancreas. NLK is also produced by T-cells activated by lectin treatment, thereby stimulating immunoglobulin secretion by peripheral blood mononu-

clear cells.[224] Here, NLK is not directly mitogenic, but instead acts early in the response which culminates in antibody-producing cells, with continued production of immunoglobulin by differentiated cells being NLK-independent. This pattern of stage-specific effect is reminiscent of NLK's effects on neurons, and it was suggested that NLK's action as a survival factor is probably limited to a critical period during development and that subsequent to this stage, NLK may impact particular aspects of neuronal function, but it is not directly required for continued viability of the NLK-responsive neuronal subpopulation.[222]

It is significant to note that biologically active factors which promote the survival of cultured spinal neurons and exhibit relative molecular masses of 50 to 60 KDa have been identified from *in vitro* preparations of muscle,[237,238] as well as fetal bovine serum.[239] Furthermore, B-lymphocyte growth factors of 50 to 69 KDa have also been described,[240] consistent with NLK's effects as a lymphokine. Therefore, it is plausible that this factor may have been implicated in numerous systems via various experimental approaches prior to its molecular identification as one molecule.

Befitting a molecule that would separately be described as an autocrine motility factor involved in tumor invasion and metastasis, serum PHI levels were defined as a marker of "substantial value as an index of growth of metastatic mammary carcinoma" as early as 1954.[226] Indeed, PHI levels serve as tumor progression markers for patients with malignant tumors including gastrointestinal, kidney, colorectal and lung,[241-244] and similar to AMF, PHI has been suggested as a urinary marker for bladder cancer.[245] Additionally, it was proposed that serum levels of PHI served as a significant indicator of various diseases including, but not limited to tumors of various organs which were metastatic to the liver.[246] Potentially this finding was due to enhanced cellular metabolism and necrotic cellular lysis, however the fact that other glycolytic as well as nonglycolytic housekeeping enzymes exhibited a significantly lower correlation with the presence of disease suggested a more complex explanation. In light of recent findings, enhanced levels of PHI may instead have represented secreted cytokine deposited into the serum from leaky tumor vasculature, or possibly a combination of both mechanisms. Indeed, the PHI promoter shows structural similarities to both housekeeping and facultative gene promoters, providing a potential mechanism of PHI upregulation dictated by its multifunctional nature as both a glycolytic enzyme and a trophic extracellular mediator.[247]

It has been suggested that NLK binds to the surface of cells in a carbohydrate-dependent manner utilizing a PHI-like structure and that NLK may act in a lectin-like fashion, recognizing sugar-containing molecules at the neuronal cell surface.[221] This proposal was based on the fact that active PHI is a dimer, while monomers are capable of binding substrate but are enzymatically inactive. The potential lectin-like mechanism of NLK could explain migration stimulation by AMF if the activity of AMF relies upon microclustering of gp78 on individual cells, implying that any cross-linking effects would to take place within the plane of the membrane on isolated cells, as cell-cell interactions are eliminated in the phagokinetic migration assay, and gp78 is already clustered on the cell surface in macroaggregates.[192,196,197,205]

The possibility that AMF exerts its effects through a lectin-like activity cannot be ruled out at this time, however, original description of NLK as PHI prompted the proposal that the enzymatic activity of PHI might account for the biological responses observed in reports on NLK function.[225,230] As AMF-R represents a glycosylated transmembrane receptor, and since carbohydrate-phosphate inhibitors which inhibit PHI's enzymatic activity also block AMF's ability to stimulate cellular migration, it is plausible that the signaling of AMF/NLK/PHI may be initiated by direct interaction with the carbohydrate side chains of the AMF receptor, gp78. Evidence against this proposal comes in the form of monoclonal antibodies which did not inhibit PHI enzymatic activity but did, however, block both NLK's ability to induce immunoglobulin secretion by activated mononuclear cells as well as promote survival of spinal and sensory neurons which were unaffected by NGF.[225]

The third plausible explanation for the extracellular exertion of AMF effects is that the PHI polypeptide, or some processed version of it, may be an ligand for a cell surface receptor.[225,230] Thus, the identification of AMF as NLK/PHI implies that, based on the above evidence, this pathway is dependent upon a single AMF receptor, gp78, and that the inhibition of AMF activity by carbohydrate phosphates probably represents an issue of steric hindrance. Although AMF may indeed bind to gp78 through the extracellular sugar moieties of this transmembrane molecule, the active secretion of NLK by cells transfected with an expression construct of PHI,[222,225] as well as the presence of altered glycosylation of gp78 which is capable of responding to AMF in cells selected for enhanced metastatic ability and the capacity of normal 3T3-A31 cells to express functional AMF receptor suggest that binding of AMF to its receptor is not merely a case of a displaced cytosolic enzyme finding a molecular mimic for its substrate within available glycoprotein side chains. Indeed, this supposition would still require that such anomalous sugar recognition somehow activate the intrinsic properties of the transmembrane molecule which harbors the recognition residues.

The importance of sugar moieties in tumor invasive processes is not a new concept and numerous reviews on the subject are available.[248,249] Aside from the obvious importance of lectin-mediated events in tumor cell inter-

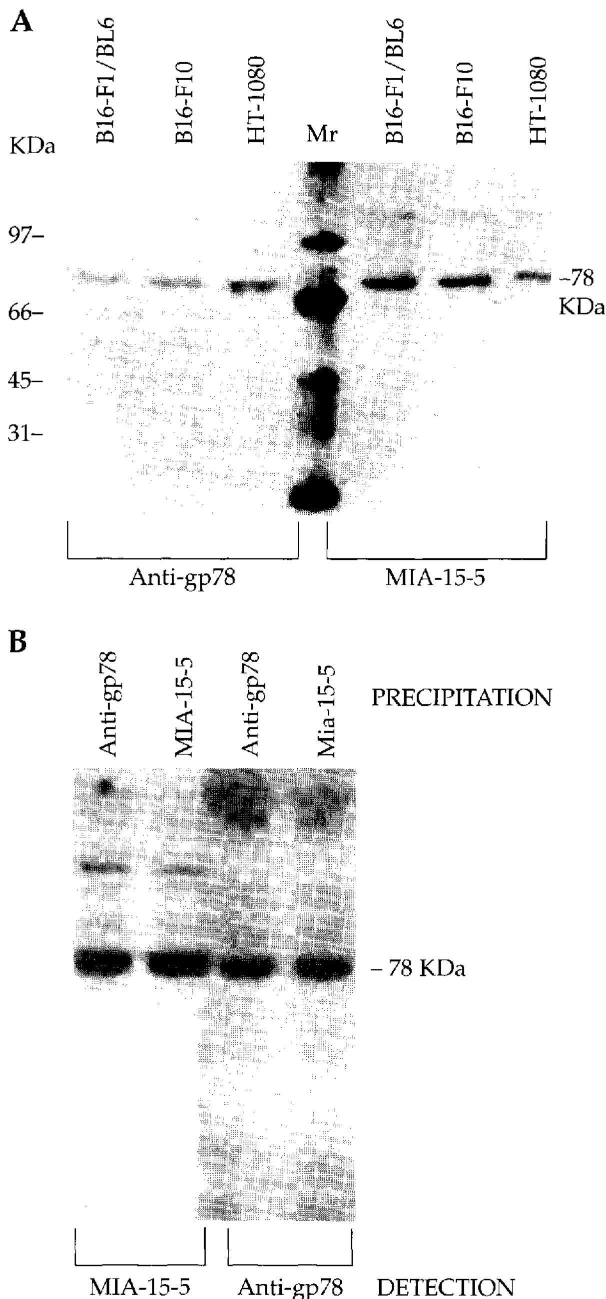


*Figure 1. Recognition of gp78 by the MIA-15-5 motility inhibiting antibody. (A) Immunoblotting comparison of the staining patterns of the anti-gp78 and the MIA-15-5 mAb's in B16-F1/BL6 and B16-F10 murine melanoma as well as HT1080 human fibrosarcoma cell lysates. M, molecular weight protein standards shown in KDa at the left. (B) Immunoblot detection of SDS-PAGE-separated anti-gp78 mAb and MIA-15-5 mAb immunoprecipitates. Proteins were immunoprecipitated from whole cell lysates with one or the other antibody (PRECIPITATION), separated by SDS-PAGE, and immunoblotted (DETECTION) with the opposite antibody as well as with the precipitation antibody as a control. 78KDa shows the migration of gp78 based on protein molecular weight markers (not shown).*

actions with their environment[250] and HA-adhesive events mediated by the cell surface glycoprotein CD44 described above, numerous examples of sugar-specific effectors of cellular locomotion have been described. Previous studies demonstrated that cell migration could be inhibited by treatment of normal or tumor cells with an antibody which recognized N-linked galactosyl or mannosyl residues, alone or in complexes, which preferentially bound two major species of membrane-associated antigen.[251] In addition, membrane glycoproteins are preferentially anchored to the cytoskeleton at the leading edge of the lamella in a manner which suggests force application towards cellular motility.[252] Similarly, the lysosome-associated membrane glycoprotein LAMP-1 is present on unique cell surface domains involved in cell migration, and altered glycosylation of LAMP-1 in transformed cells has been postulated to contribute to their ability to disseminate during tumor invasion.[253] An anti-tumor antibody which binds to the sialyl Lewis (y) antigen [Le(y)], a carbohydrate determinant widely expressed on human carcinomas, recognizes LAMP-1 and was shown to suppress cellular migration. The altered glycosylation of LAMP-1 in invasive cells is reminiscent of the case of the AMF receptor, gp78, and it is interesting to note that expression of Le(y) decreases in breast carcinoma cells grown to confluence, whereas stimulation of their migration induces reexpression of the Le(y) antigen,[254] suggesting that glycosylation-dependent modulation of migration mediating effectors on the cell surface may be a common theme, at least in tumor biology. In support of this contention, the carbohydrate antigen sialyl Le(x) appears to be involved in colorectal cancer metastasis[255] and Le(a) expression has recently been described as a prognostic indicator of disease recurrence in gastric cancer .[256].

Analogously, a monoclonal antibody termed MIA-15-5 (for migration-inhibiting antibody), which was generated against a specific carbohydrate structure present in the blood group antigen precursor H-antigen and its related structures (Fuc1-2Gal1-R carrier carbohydrate), reacted preferentially with high- but not low- metastatic melanoma cells.[257] This antibody inhibited the migration and experimental metastasis of high-metastatic murine melanoma cells *in vivo* without apparent effect on cell proliferation, and was shown to react with four antigens in human lung adenocarcinoma MAC-10 cells, one which exhibited an apparent molecular mass similar to gp78. Immunoblotting and immunoprecipitation/blotting procedures were used to determine that this antibody does indeed recognize the carbohydrate side chain of the AMF receptor (*Fig. 1*), however phagokinetic migration analysis utilizing MIA-15-5 in conjunction with components of the AMF system demonstrated that although this antibody was able to inhibit the basal migration of highly metastatic B16-F10 murine melanoma cells, the heightened loco-

motion of these cells in response to AMF or the AMF-mimicking anti-gp78 monoclonal antibody was unaffected by MIA-15–5 treatment (*Table 1*). This data suggests that the carbohydrate-recognizing MIA antibody was incapable of preventing both ligand-binding and signal transduction following receptor perturbation. These findings support the theory that AMF's interaction with gp78 is that of a *bone fide* ligand with its receptor and suggest that antimetastatic therapy via an immunotherapy approach which utilizes the MIA-15-5 antibody may not be as successful as predicted by the original analysis of unstimulated melanoma cells in isolation, at least with regard to malignant tumors which utilize the AMF system. Interestingly, immunohistochemical analysis of samples from patients with lung carcinoma with the MIA-15-5 antibody demonstrated an inverse correlation between antibody positivity and patient survival, a finding which highlights the utility of AMF receptor expression in prognostic analysis.[258]

The presence and indeed the acquisition of new functions by cytosolic enzymes in the extracellular millieu as described here for the glycolytic enzyme PHI as a trophic factor which is also capable of inducing cellular locomotion is not unprecedented. Comparison of the autocrine mechanisms promoting migration in metastatic melanoma cells and ras-transfected fibroblasts revealed a second autocrine migration-stimulating factor which was distinct from AMF and was dubbed autotaxin (ATX).[259] This molecule exhibited markedly different physical characteristics in addition to utilizing a wholly unique signal transduction system from AMF, despite the fact that its motility-stimulatory effects also appear to be mediated by a cell surface receptor. In addition to representing an additional member of the autocrine locomotory factor group, identification of autotaxin's molecular nature revealed it to be another member of the ecto/exoenzyme family. Autotaxin represents an alternatively-spliced extracellular form of phosphodiesterase I (PD-I), a member of the nucleotide pyrophosphatase family.[260] Although the distribution of ATX appears to be more restricted than that of AMF, recently ATX has been implicated in regulation of neuroblastoma cell migration,[261] indicating that it too may play a role in tumor invasion *in vivo* and suggesting that the expression of ATX or its receptor (upon identification) may serve as a useful, clinically relevant prognostic tool. Although this factor is an N-linked glycoprotein, the carbohydrate moieties are not required for its stimulation of cell migration,[262] therefore, since the receptor for ATX has yet to be determined, it is not possible at this stage to postulate a mechanism by which ATX interacts with its receptor, although it is likely through a specific ligand:receptor interaction as suggested for AMF.

More recently, the previously described platelet-derived endothelial cell growth factor (PD-ECGF), which represents the sole angiogenic activity present in platelets, was identified as the intracellular enzyme thymidine phosphorylase (TP), which catalyzes the reversible conversion of thymidine to deoxyribose-1-phosphate and thymine.[263] Interestingly, although this molecule is active as a homodimer similar to PHI/NLK,[264] it does not appear to act via a receptor-mediated pathway,[265] and unlike the PHI/NLK system, enzymatic activity is required for TP to exert its angiogenic effect.[266] It is significant to note, however, that the expression and activity of this enzyme correlates with disease progression in cancers of the urinary bladder, lung, pancreas, breast, as well as tumors of the colorectal and gastrointestinal systems,[267-273] suggesting that a general property of members of this new class of ecto/exoenzymes may be their presence as prognostic indicators if not critical modulators of tumor malignancy.

In addition, other cases of intracellular molecules associated with extracellular function have been described which bear particular significance to the processes of migration and invasion. Glyceraldehyde 6-phosphate dehydrogenase (GAPDH) levels have been correlated with the propensity for spontaneous migration in sublines of the Dunning R-3327 rat prostatic adenocarcinoma, a system in which motility directly correlates with experimental metastasis.[274] In addition, a naturally-occurring, liver-derived 21-amino acid fragment (dubbed Invasion Inhibiting Factor-II) of the high mobility group protein 17, a DNA-binding non-histone protein, interacts with a cell-surface component and thereby suppresses motility of tumor cells of various lineage (including that induced by AMF) and inhibits invasion of ECM barriers following arrest of tumor cells in the capillary bed of the lung *in vivo*,[275] effectively blocking experimental and spontaneous metastasis. Thus this polypeptide, normally found within the cell nucleus, has dramatic effects on phenotypic parameters important for tumor invasion when expressed extracellularly.

This emerging class of ecto/exoenzymes represents a novel group of apparently unrelated intracellular mole-

***Table 1.* Effect of the MIA-15-5 mAb on the motility of B16-F10 melanoma cells.**

| *Condition* | *Motility* ($\mu m^2/hr$) | *Percent Control* |
|---|---|---|
| Control | 13.5 ± 1.1 | 100 ± 8.1 |
| MIA-15-5 | 9.8 ± 0.9 | 72.6 ± 6.7[a] |
| B16-F1 AMF (50 pg) | 25.4 ± 2.7 | 188 ± 20[a] |
| AMF + MIA-15-5 | 25.1 ± 2.7 | 186 ± 20[a] |
| anti-gp78 mAb | 33.6 ± 2.4 | 249 ± 18[a] |
| anti-gp78 + MIA-15-5 (1:1)[b] | 31.1 ± 3.3 | 230 ± 24[a] |

[a] $p<0.0001$ from control by two-tailed Student's T-test
[b] MIA-15-5 in a 5:1 excess had a similar lack of effect

cules which may have important effects on cellular processes involved in tumor invasion and metastasis. It is likely that with continued investigation into tumor-associated processes, more members of this family will be identified, as tumor processes often represent the distortion of normal processes via inappropriate use of molecules for disparate functions or to provide inappropriate signals which are beneficial for survival of the tumor cell.

### *Conclusions*

Cellular motility is a critical component of numerous physiological processes and although all components are necessary for the regulated choreography of this activity, as with many tumor-related mechanisms, the distortion of a regulated process through disruption or alteration of key players (eg. protooncogenes) aids in progression of the tumor progression the invasive and metastatic phenotype. The interconnecting roles of cell surface receptors, soluble factors and the extracellular matrix, in addition to their respective signaling pathways are so inter-reliant upon one another that shifts in the natural balance of these components can have dramatic effects. Such is the case with AMF, an intracellular enzyme (PHI) with proliferative effects as an extracellular cytokine in a regulated system (NLK), as well as invasive and metastatic effects on various tumor types upon disregulation of the expression of itself (AMF), its receptor (gp78/AMF-R), or its signaling components (12-LOX, transcription factors). It is unlikely that this new class of ecto/exoenzymes is limited to the examples described here, and the identification of these and other related molecules will likely yield potential tools for both prognostic evaluation and tumor-specific treatment strategies.

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