Journal of Neuro-Oncology **18**: 139–149, 1994.

Malignant melanoma metastasis to brain: role of degradative enzymes and responses to paracrine growth factors

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Key words: melanoma, metastasis, proteases, endoglycosidases, growth factors, growth factor receptors, basement membrane, blood brain barrier, neurotrophins

Abstract

Mouse and human melanoma cells metastatic to the brain express degradative enzyme activities that are used for invasion of brain basement membrane and parenchyma. Compared to poorly metastatic or lung- or ovary-metastatic murine melanoma lines, the brain-metastatic sublines secreted higher levels of a variety of degradative enzymes. Brain-metastatic murine and human melanoma cells also degraded subendothelial basement membrane and reconstituted basement membrane at rates higher than other metastatic melanoma cells. In some cases these degradative activities in mouse and human melanoma cells can be induced by paracrine factors known to be present in the brain parenchyma, such as nerve growth factor (NGF). NGF stimulates the expression of degradative enzymes, such as the endo-β-glucuronidase heparanase, that are important in basement membrane penetration but this factor does not stimulate melanoma cell growth. The growth of brain-metastasizing melanoma cells appears to be stimulated by other paracrine growth factors, such as paracrine transferrin. Melanoma cells metastatic to brain express higher numbers of transferrin receptors and respond and proliferate at lower concentrations of transferrin than do melanoma cells metastatic to other sites or poorly metastatic melanoma cells. The results suggest that degradation and invasion of brain basement membrane and responses to paracrine neurotrophins and paracrine transferrins are important properties in brain metastasis of murine and human malignant melanoma cells.

Introduction

The formation of brain metastases is usually the final, fatal phase of the progression of many solid cancers, including melanoma, breast cancer, lung cancer and other malignancies. The metastatic process consists of a series of very complex, sequential events involving specialized subpopulations of tumor cells, and unfortunately it occurs often in cancer patients with malignant melanoma. All metastatic cells that colonize distant organ sites must escape from the primary tumor by local invasion, enter the circulation, circulate in the blood, adhere to and exit from the microvasculature in a distant or-

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gan, and grow in response to the local microenvironment at the new site [1, 2], but there are several significant differences between the development of metastatic lesions in the central nervous system (CNS) and other organ sites. Metastatic cells in the CNS must adhere to brain microvessels, penetrate the blood-brain endothelial barrier (BBB) and grow in the brain parenchyma [2–4].

The brain microvessels represent an exceedingly small fraction of the total microvasculature and, in general, the tumor cells that gain access to the brain must traverse microvessels at other organ sites, thus the process of brain metastasis formation is generally thought to be nonrandom. Clinical and animal studies have demonstrated that the distributions of distant metastatic lesions in many cancers are nonrandom, with certain cancers exhibiting a propensity to colonize particular organs [2, 3, 5]. Such observations lead Paget [6] to describe the nonrandom pattern of metastatic disease as one based on the properties of the unique tumor cell 'seeds' as well as the organ microenvironment or 'soil'. This hypothesis, in which the host environment plays an equally important role as the tumor cells in the development of distant metastatic lesions, is particularly applicable to metastatic lesions in the CNS, where different tumor cell types exhibit preferential colonization of different regions of the brain [7].

The preferential colonization of particular organs or regions of organs implies that specific tumor-host cell interactions mediated by particular sets of properties and factors, such as those involved in malignant cell adhesion, invasion, and growth, are critical to the development of metastases at certain sites [1–3]. In malignant melanoma the majority of patients with advanced disease possess brain or leptomeningeal metastases [8–10]. At autopsy more than 70–90% of these patients have brain metastases [11, 12]. Palliation of brain metastases is possible, but effective treatments are rare [12, 13].

Access to the brain is tightly regulated by the BBB that controls the flow of materials into the brain. The BBB is a physiologic and anatomic structure that is mainly defined by tight junctions between the brain endothelial cells, a relatively thick basement membrane, and an underlying layer of astrocytes that strictly regulate the flow of ions, nutrients, and cells into the brain. Therefore, for brain metastasis to occur, malignant cells must adhere to the luminal surface of brain microvessel endothelial cells and then invade the BBB formed by the endothelial cells, basement membrane and astrocytes. This requires the expression of a variety of cell surface receptors and degradative enzymes.

The vast majority of brain metastases are established via blood-borne routes, with the rare exception of direct invasion of head and neck tumors or passage via the cerebral spinal fluid resulting in diffuse leptomeningeal metastases. The distribution of metastases in the brain is divided between the leptomeninges and meninges (surface of the brain) and the parenchymal interior (cerebrum, cerebellum, and brain stem), roughly paralleling brain blood flow, with about 80% of the parenchymal lesions presenting in the supratentorial regions and 15% in the cerebellum. However, different cancers exhibit differing propensities to colonize separate regions of the brain [7–12].

To study the phenomenon of brain metastatic selectivity, animal metastatic models have been developed that mimic clinical disease and show preferential tumor development in particular organs. A number of model systems representing various primary cancers have been developed to examine the metastatic cascade; however, relatively few have been developed to examine brain metastasis [2]. Using a mouse B16 melanoma cell line, Brunson *et al.* [14] and Miner *et al.* [15] selected B16 variants that exhibited brain colonization. This was performed by repeated cycles of *in vivo* selection by intracardiac inoculation, harvesting of brain tumors, and *in vitro* proliferation of the selected cells. The selection was most successful for brain meninges colonization. The selected lines B16-B14b and B16-15b, selected fourteen- or fifteen-times, respectively, for brain meninges tumor formation were shown to initially colonize the dura mater and leptomeninges, eventually invading into the brain parenchyma [16]. The B16-B14b cells were observed to form small tumor lesions in the brain, whereas the B16-B15b cells formed relatively large tumors that were deeply pigmented. Histological and electron microscopic evaluations of the tumors re-

vealed that the B16-B14b cells elicited extensive infiltrations of lymphocytes that were absent in the B16-B15b cell lesions. Removal of the spleen prior to injection of the tumor cells increased the tumor growth of the B16-14b cells, suggesting that host-tumor interactions, in this case the ability to elicit an immune response, plays an important role in the development of B16 metastatic brain lesions [17]. The selection for enhanced brain colonization did not result in selection of a dominant phenotypic characteristic of the cells, because the *in vitro* culture of various clones derived from the brain-selected B16-B14b line lost their preference for brain colonization upon prolonged culture *in vitro*. The loss of brain colonization properties of the B16-B14b cell clones was prevented if three of the unstable clones were co-cultured together, suggesting that individual tumor cell clones are stabilized by interclonal interactions [15].

To deliver melanoma cells directly to either the brain parenchyma or the meninges Schackert and Fidler [18] injected murine K-1735 melanoma cells via the internal or external carotid artery of C_3H mice. By either route of entry, the K-1735 melanoma cells only colonized brain parenchyma, indicating that K-1735 cells do not colonize the brain by random arrest and growth of cells. They then repeated this experiment with B16 melanoma cells and found similar to previous results [16,17] that the B16 cells only colonized the meninges and ventricles, indicating that the formation of brain metastases is not simply the result of nonspecific lodgment of tumor cells in the first microcirculatory network encountered [18]. In addition, they showed that the colonization of particular regions of the brain was not dependent on the initial distribution of tumor cells in the brain, but rather the interaction between host and tumor cells at specific regions. The initial arrest of all of the cell lines was demonstrated to be the same. This emphasizes that the successful brain-metastatic cell must penetrate the endothelium and BBB and proliferate in the surrounding tissue after arrest in the brain microvascular. Schackert *et al.* [19] also inoculated human melanoma cells directly into the brains of nude mice. The various melanoma cell lines all exhibited the ability to grow in the brain parenchyma after direct intracerebral inoculation. However, each melanoma line exhibited a distinct distribution in the brain after injection into the carotid artery. Interestingly, the melanoma cell lines originally obtained from human brain parenchymal lesions exhibited a propensity to colonize the CNS, while the melanoma cells derived from lymph node or subcutaneous lesions preferentially colonized the meninges. Not all brain-colonizing melanoma cells have been selected *in vivo*. Ishikawa *et al.* [20] obtained human MeWo variant lines metastatic to brain of nude mice by selection for resistance to wheat germ agglutinin *in vitro*. The brain-colonizing MeWo 70W subline was obtained by exposure to stepwise increases in concentration of wheat germ agglutinin. Here we report on the basement membrane degradative properties, invasive and growth responses to paracrine growth factors of mouse and human brain-metastatic melanoma variants.

Materials and methods

Cells

Murine brain-colonizing B16 melanoma cell lines were grown *in vitro* in Dulbecco-modified Eagle's (DME) medium without antibiotics and containing 5% fetal bovine serum (FBS) and 1% non-essential amino acids [21]. Human MeWo melanoma cells and the brain-colonizing subline 70W [20] were grown in a 1:1 mixture of DME and F12 medium supplemented with 5% FBS [22]. Bovine aortic endothelial cells and murine brain microvascular endothelial cells were established and grown as described previously [23].

Degradation of subendothelial matrix, Matrigel invasion and enzyme assays

Degradation of [^{3}H]leucine-labeled bovine aortic endothelial cell or brain microvessel endothelial cell subendothelial matrix were assayed according to Nakajima *et al.* [24]. Heparanase activity was determined by the degradation of radiolabelled heparan sulfate (HS) using high speed gel permeation

chromatography [25]. The cysteine proteinases cathepsin B and cathepsin H were measured with a fluorescence assay at 37° C using the peptide methylcoumarylamide (MCA) derivatives Z-Arg-Arg-MCA and Arg-MCA at pH 6.2 and 6.5, respectively (both from Peninsula Laboratories, Belmont, CA) [26]. Cathepsin D was measured at pH 3.6 using acid-denatured hemoglobin as a substrate at 45° C [27]. Type IV collagenase was measured using [^{3}H]proline-labeled type IV collagen according to Nakajima *et al.* [28].

The reconstituted basement membrane invasion assay was modified from the procedures of Albini *et al.* [29]. We used Transwell (Costar) cell culture chambers and monitored invasion by a fluorescence plate scanner (Cytofluor, model 2300, Millipore) [22, 30]. Matrigel (Collaborative Research) was diluted 1:30 in cold DME/F12 medium without phenol red and applied (20 µg/100 ml/filter) onto the upper surface of the Transwell filter (6.5 mm diameter, 8 µm pore size) insert. The lower chamber contained a mixture of 0.5% low gelling agarose (FCM Bio Products), 0.5% gelatin (Sigma) with addition of 2.5–10 nM of the chemotactic peptide N-formyl-Met-Leu-Phe (Sigma) and/or 25% phenol red-free fresh human brain endothelial cell-conditioned medium [22, 30]. Cells were suspended in serum-free, phenol red-free DME/F12 medium and seeded into the upper compartment of the Transwells at 2.0×10^4 cells/filter. After 72 h incubation at 37° C with varying amounts of purified, biologically active 2.5 S murine NGF (0–100 ng/ml), the upper chambers were carefully removed and the bottom chambers treated with 1 µM calcein-AM for 1 h at 37° C. After several washes the plates were analyzed for fluorescence using the Cytofluor fluorescence plate scanner at 530 nm. The accuracy of the invasion assay was assessed by fluorescence microscopy and replicate determination of cell number by measuring specific fluorescence of the lower side of the Transwell filters and removal of filter-bound cells by trypsin-EDTA treatment as described previously [22, 30].

Binding of ^{125}I-labeled transferrin

Murine or human transferrin (Tf) at 5 mg/ml in phosphate-buffered saline (PBS) was saturated with iron by incubation with ferric ammonium citrate (0.1 mg/ml) in 0.01 M $NaHCO_3$ for 4 h at room temperature. Unbound iron ions were removed by dialysis against phosphate-buffered saline (PBS) overnight. Iron-saturated Tf was labeled with ^{125}I according to Inoue *et al.* [31]. The specific activities of ^{125}I-labeled Tf ranged from 60 to 65 cpm/fmol. Confluent cell monolayers were incubated with ^{125}I-labeled Tf for various times at 4° C washed with PBS, and cell-associated radioactivity was determined [31]. Nonspecific binding of ^{125}I-labeled Tf was estimated by the amount of labeling in the presence of a 200–1,000-times excess concentration of unlabeled Tf [32].

Effects of nerve growth factor and transferrin on cell growth

Cells were plated at 1,000 cells/well in 96 well plates or at 5,000 cells/well in 24 well plates in media containing 1% [v/v] FBS. After 24 h, the medium was changed to that containing 0.3% or 0% [v/v] FBS and increasing amounts of holo mouse Tf (for the murine cell lines) or human Tf (for the human cell lines) was added. After 3–6 days, the cells were enumerated using a crystal violet cell quantitation assay [33], or the cells were removed from plates with trypsin and counted with a model ZM Coulter Counter. Alternatively, a fluorescence cell viability assay was used to measure the effects of murine 2.5S NGF (Collaborative Research, Bedford, MA) on cell growth [22, 30]. Briefly, cells (6.6×10^3/well) in 96-well plates were exposed to 1.17 µM calcein-AM (Molecular Probes, Eugene, OR) for 45 min at 37° C. Calcein-AM hydrolysis by intracellular esterases yielded the intensely fluorescent product calcein. Following incubation in 100 µl volume, the cells were rinsed gently with 1000 volumes of PBS without Ca^{2+} or Mg^{2+} or pH indicator. After sequential washes, 100 µl of PBS without Ca^{2+} or Mg^{2+} or pH indicator was added, and the plates were scanned using a Cytofluor model 2300 (Millipore)

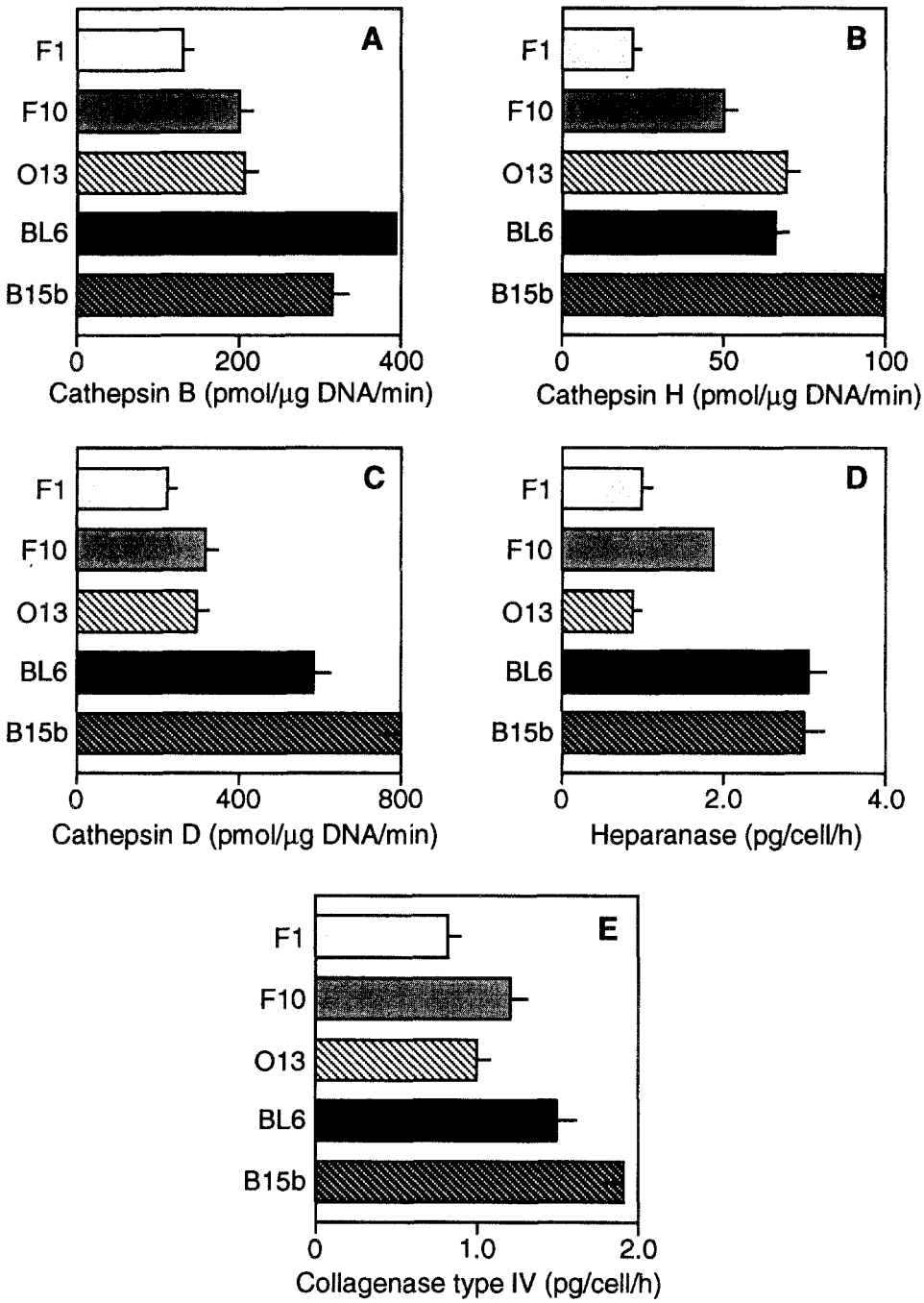

Fig. 1. Degradative enzymes produced by murine B16 melanoma sublines in vitro. **A,** cathepsin B; **B,** cathepsin H; **C,** cathepsin D; **D,** heparanase; **E,** type IV collagenase (see Methods).

with emission filters set to detect calcein fluorescence (530 nm) as described previously [22]. This assay was linear and sensitive down to 100 cells/well [22].

Results

In the absence of growth factor stimulation in culture highly brain metastatic murine melanoma cells secrete high levels of degradative enzymes. For ex-

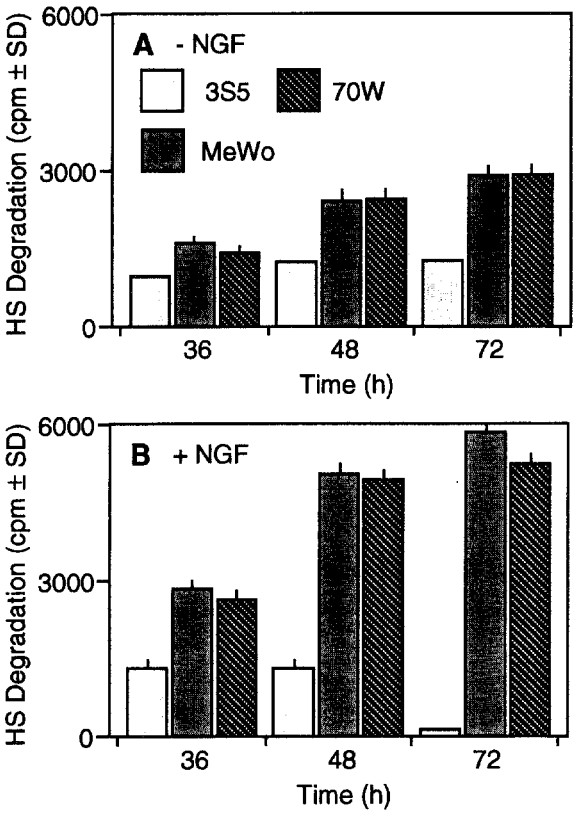

Fig. 2. Effect of 2.5S NGF on the production of heparanase by human MeWo melanoma cells. **A,** HS degradation in the absence of NGF; **B,** HS degradation in the presence of 50 ng/ml (~ 2 nM) 2.5S NGF (see Methods).

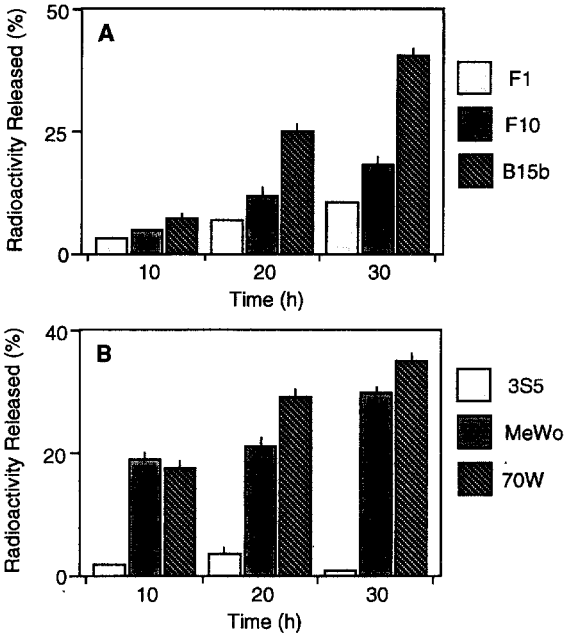

Fig. 3. Degradation of radiolabeled subendothelial matrix by murine B16 and human MeWo melanoma cells. **A,** murine B16 melanoma sublines; **B,** human MeWo melanoma sublines (see Methods).

ample, brain-colonizing B16-B15b cells secreted the highest levels of cathepsin H, cathepsin D, heparanase and type IV collagenase (Fig. 1). Similar results were found with human MeWo melanoma lines. The brain-metastatic 70W subline secreted high concentrations of degradative enzymes [22, 30]. For example, the metastatic human melanoma lines MeWo and 70W degraded significantly higher amounts of heparan sulfate than the poorly metastatic human 3S5 line derived from the MeWo melanoma line (Fig. 2A), indicating their release of basement membrane HS-degrading heparanase [24]. When tested for their abilities to degrade subendothelial matrix, the highly brain metastatic murine B16-B15b and human 70W cells solubilized radiolabeled subendothelial matrix at higher rates than the other melanoma lines (Fig. 3).

We next determined whether brain-colonizing murine and human melanoma cells could invade reconstituted basement membrane (Matrigel). In the absence of stimulation by neurotrophic factors, the brain-metastasizing murine melanoma lines invaded Matrigel at higher rates than melanoma lines that did not colonize brain (Fig. 4A). This result was not found with human melanoma lines (Fig. 5A). When 2.5S NGF was added to melanoma cells growing on extracellular matrix in culture [22, 30], there was an increase in Matrigel invasion, particularly of the murine and human brain-metastatic melanoma lines (Figs 4B and 5B). The NGF-stimulated increase in the rate of invasion of Matrigel paralleled the increase in degradative enzyme secretion by the brain-metastatic melanoma cells treated with NGF (Fig. 2B), suggesting a role for NGF in brain metastasis.

In addition to invasion, we examined the ability of murine and human melanoma sublines to grow in response to paracrine growth factors [2, 31] and express receptors for these factors [22, 30–33]. We had previously found that tumor cell growth response to and their ability to bind transferrin correlated with metastatic capability in metastatic tumor models [2, 31–33]. We therefore examined the mitogenic prop-

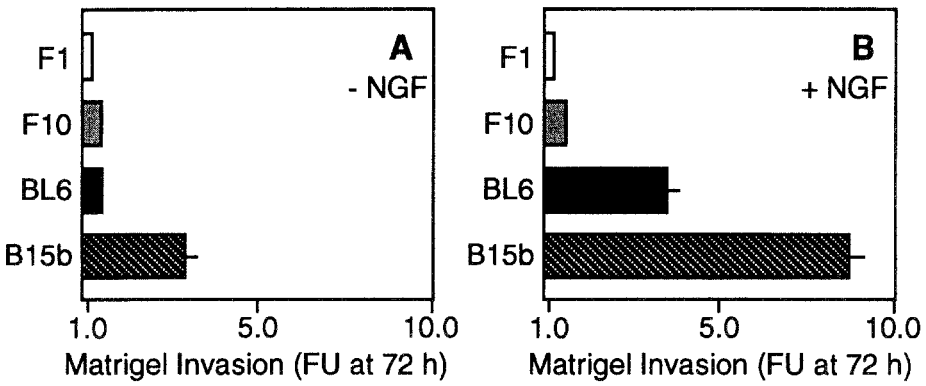

Fig. 4. Effect of 2.5S NGF on invasion of murine B16 melanoma variant cells through a Matrigel reconstituted basement membrane. **A,** invasion in the absence of NGF; **B,** invasion in the presence of 50 ng/ml 2.5S NGF (FU, fluorescent units; see Methods).

erties of NGF and transferrin in cultures of murine and human melanoma cells. Addition of various concentrations of NGF to murine or human melanoma cells resulted in a reduction in growth rate in the cell lines. For example the growth rates of the human melanoma lines were reduced by approximately 20% in the 70W and MeWo lines but less in the 3S5 line after treatment with 2.5S NGF [22], whereas addition of transferrin to melanoma cells resulted in growth stimulation [31–33]. Examination of the responses of various murine (Fig. 6A) and human (Fig. 7A) melanoma cell lines to transferrin in the absence of FBS indicated that the brain-metastatic sublines responded best to transferrin and generally expressed the highest numbers of transferrin receptors (Figs 6B and 7B), suggesting that transferrin response may be an important property of brain-metastatic melanoma cells.

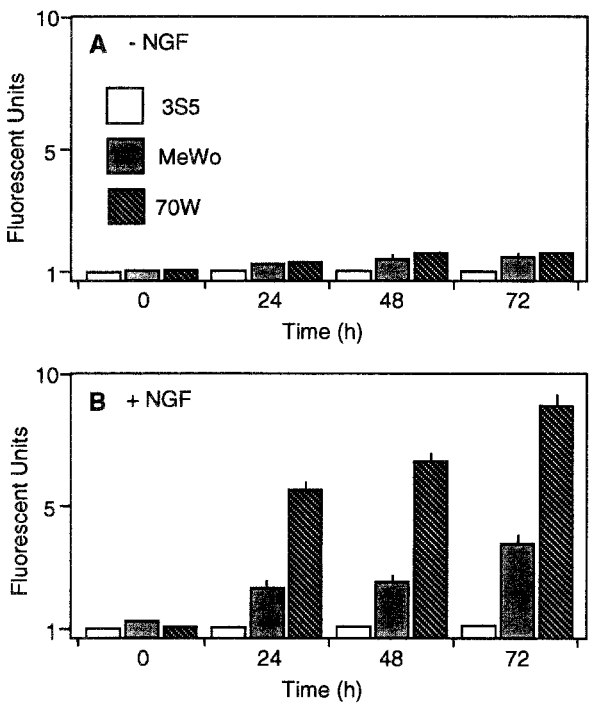

Fig. 5. Effect of 2.5S NGF on invasion of human MeWo melanoma variant cells through a Matrigel reconstituted basement membrane. **A,** invasion in the absence of NGF; **B,** invasion in the presence of 50 ng/ml 2.5S NGF (see Methods).

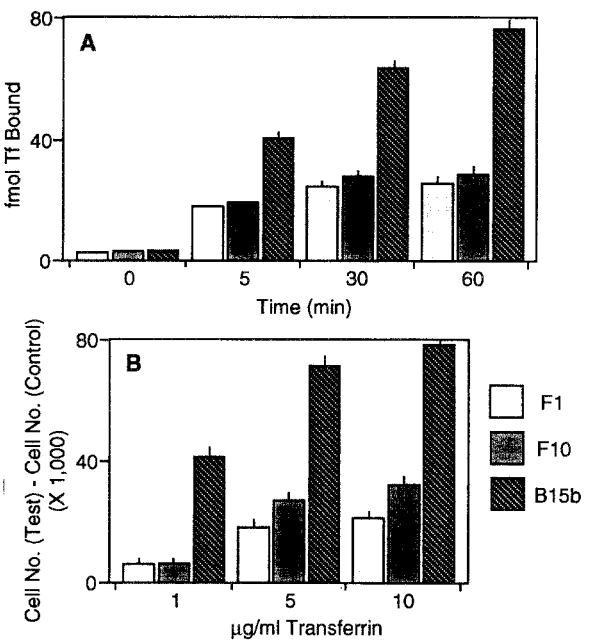

Fig. 6. Binding of ^{125}I-labeled transferrin to murine B16 melanoma variants and their growth responses to transferrin. **A,** time course of binding of ^{125}I-labeled transferrin; **B,** growth response to transferrin in the presence of 0.3% FBS at 72 h (see Methods).

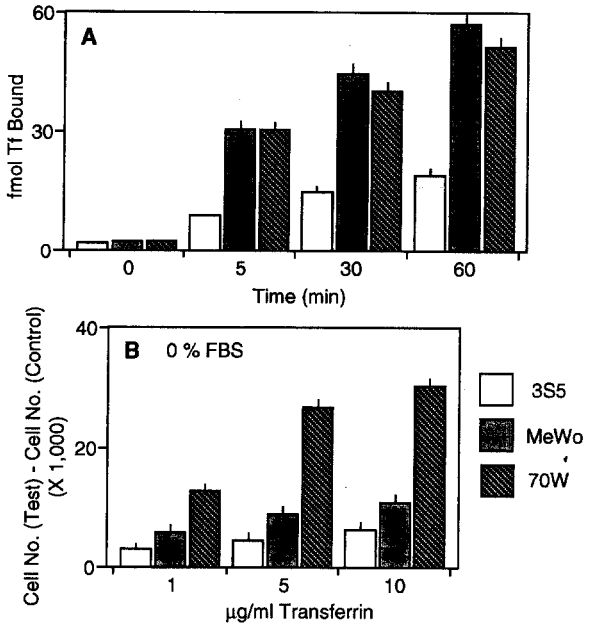

Fig. 7. Binding of ^{125}I-labeled transferrin to human MeWo melanoma variants and their growth responses to transferrin. **A,** time course of binding of ^{125}I-labeled transferrin; **B,** growth response to transferrin in the absence of FBS at 72 h (see Methods).

Discussion

There is a growing list of tumor cell and host properties that are important in the process of metastasis [1–3]. Each of these steps involves many tumor cell and host molecules, and metastasis to brain probably involves some unique characteristics of tumor cells that may not be necessary for metastasis to other organ sites. The initial interactions of circulating malignant cells with host organs occurs in the microcirculation. Using cell adhesion assays the interactions between melanoma cells and microvessel endothelial cells was investigated and shown to be related to brain metastasis. For example, B16 melanoma sublines of high brain colonization potential adhered at higher rates to brain-derived microvessel endothelial cell monolayers *in vitro* than lines that failed to colonize the brain [2, 34]. Similar results have been obtained with the human MeWo melanoma system (unpublished data). Several studies have examined the ability of specific organ-colonizing tumor cell variants to adhere to microvessel endothelial cells derived from various organs, and in general, these have shown that tumor cells of high metastatic potentials usually adhere at higher rates to target endothelial cells than poorly or nonmetastatic cells [reviewed in 2]. Furthermore, the preferential adhesion of organ-colonizing tumor cells has been demonstrated with endothelial cells derived from small but not large blood vessels from the appropriate organ. Thus the B16-B15b cell subline selected for brain colonization adhered at the higher rates to brain-derived microvessel endothelial cells than the B16 sublines selected for lung or ovary colonization [2, 34]. The adhesion of brain-metastatic malignant cells to the appropriate microvessel endothelial cells suggests that microvessel endothelial cells display organ-specific adhesion molecules. Indeed, studies with animal tumor metastatic models indicate that differential quantitative expression of endothelial cell surface proteins on organ microvessels [35]. Using in situ radiolabeling of the microvasculature and cultured cells isolated from various murine organs, Belloni and Nicolson [36] showed the preferential expression of particular proteins in the microvessels of different organs. Such studies indicate that endothelial cells from specific organs display different cell surface determinants, and some of these differentially expressed proteins probably play a role in organ specific cellular adhesion.

Another important property of brain-metastasizing tumor cells is the ability to penetrate the brain vasculature and BBB. This requires the production of degradative enzymes that solubilize the BBB and its basement membrane. Tumor cells that possess the ability to colonize the brain express high levels of basement membrane hydrolytic enzymes, such as type IV collagenase and heparanase [24]. Some of these enzymes may be induced by the microenvironment. If the appropriate paracrine signals are received by malignant cells, they might be stimulated to release higher quantities of degradative enzymes and exhibit enhanced cell motility. We have found that brain-metastatic human melanoma cells are sensitive to NGF [22, 30]. In contrast to chronic paracrine factors in the brain, NGF is an acute paracrine factor that could be induced by invading tumor cells. NGF binds to specific receptors on the surfaces of some PC12 cells but does not induce mitogenesis; some of these receptors are of the low-

affinity type, such as $p75^{NGFR}$, and some are of the high-affinity type, such as the family of *trk* protooncogene receptors that may form molecular complexes with $p75^{NGFR}$ [37]. During progression of human melanoma there is a progressive increase in the number of $p75^{NGFR}$ receptors [38]. We have found that NGF can stimulate extracellular matrix degradation and invasion in mouse and human melanoma cells, particularly in the brain-metastatic melanoma cell lines, and it apparently does this independent of the *trkA*-encoded receptor $p140^{proto\text{-}trkA}$ [30], suggesting a role for the low-affinity NGF receptor $p75^{NGFR}$ in signaling invasion of brain-metastatic melanoma cells. Although brain-colonizing melanoma cells constitutively express high levels of degradative enzymes, incubation of these cells with the neurotrophic factor NGF increases production of matrix-degrading enzymes [22, 30]. Interestingly, this only occurs if the cells are grown on extracellular matrix, suggesting that melanoma cells must receive the appropriate matrix signals before they can respond to neurotrophic factors, such as NGF [30].

Once malignant cells have invaded brain tissue, they can proliferate but only if they can respond mitogenically to paracrine growth factors in the brain microenvironment [39]. Alternatively, metastatic cells can synthesize their own autocrine growth factors. Organs appear to contain local concentrations of paracrine growth factors that can preferentially stimulate the growth of certain metastatic tumor cells. These were first discovered by examining the ability of specific organ-conditioned media to stimulate or inhibit the growth of organ-colonizing malignant cells. For example, highly lung-metastatic melanoma cells responded best to lung-conditioned medium and grew at higher rates than when exposed to organ-conditioned media from organs that were not targets for metastasis [40]. Using this assay a lung-derived growth factor was subsequently isolated that differentially stimulated the growth of various metastatic cell lines that preferentially metastasized to the lung [41]. Recently, this growth factor was demonstrated to be related to transferrin [42]. Interestingly, brain tissue-conditioned medium failed to differentially stimulate the growth of highly brain-colonizing B16-B15b cells, suggesting that B16 brain-colonizing cells may respond to unstable or insoluble growth factors found in the extracellular matrix of brain. Alternatively, they may respond to a growth factor-poor brain environment by increasing the expression of growth factor receptors that are important for tumor cell proliferation. There is some evidence for this last possibility, because the brain appears to contain low concentrations of transferrin-like growth factors. To respond to this growth environment malignant cells may have to express increased numbers of receptors or express receptors of higher affinity for their ligands. When murine melanoma cells of varying potentials to colonize brain and other organs were examined for transferrin-like receptors, the highly brain-colonizing sublines, such as B16-B14b and -B15b, expressed the highest numbers of transferrin receptors [31]. Similarly, rat mammary adenocarcinoma cells selected to metastasize to the brain expressed the highest numbers of transferrin receptors in this system [31, 43]. Regardless of the mechanism, the end result is that brain-metastatic cells should have a growth advantage over their nonmetastatic counterparts in brain microenvironments [7]. The brain is a rich source of other growth factors, such as fibroblast growth factors (FGFs), and certain melanoma and other cancer cells have been found to use an autocrine growth loop by producing FGF and expressing its receptors [44].

Differential expression of metastatic melanoma cell adhesion receptors, motility factor receptors, degradative enzymes and growth factor receptors all suggest that brain-specific colonization may be due to a combination of quantitative and qualitative differences of gene expression and altered responses to brain environments. The identification of organ specific determinants for brain metastasis would not only be very useful for understanding the mechanism of CNS metastasis, but it might also be useful for organ-specific delivery of therapeutic agents to treat brain metastases.

Some potential molecular markers for brain metastasis have been identified. The expression of a $\sim$ 90 kD cell surface glycoprotein was correlated with the brain colonization capabilities of B16 mouse melanoma cells [15]. This glycoprotein (gp90) was originally identified as a major cell surface protein accessible to cell surface iodination

that was expressed at higher levels on the brain-colonizing B16 variants and at lower levels on lung-colonizing or poorly metastatic cells. Additionally, cell clones derived from the brain colonizing variants that lost their brain colonization propensity had decreased expression of this glycoprotein [15]. The gp90 glycoprotein was one of the first molecules whose expression correlated with preferential organ colonization of metastatic cells. Although antibodies against gp90 failed to inhibit blood-borne implantation or adhesion of brain-colonizing melanoma cells to brain microvessel endothelial cells, they did inhibit B16 cell growth [31]. Using the antibodies and immunohistochemical techniques gp90 was shown to be expressed on other tumor cell types and on various adult tissues [21], and subsequently it was shown to be a homo-dimer subunit of the 180 kD transferrin receptor. Brain-metastatic cells exhibited a greater growth stimulatory response to purified transferrin than the other malignant cells that displayed decreased amounts of transferrin receptor, suggesting that brain metastasis requires alterations in the expression of growth factor receptors, particularly transferrin receptors, and the overexpression of certain growth factor receptors may be required for a metastatic cell to respond to the microenvironments of certain organs. Similarly, overexpression of brain endothelial cell receptors, response to brain motility factors, overproduction of degradative enzymes and other factors are probably required collectively to enable malignant cells to metastasize to the brain.

A number of studies have clearly shown that metastasis, and particularly metastasis to the CNS, requires a complex series of steps and interactions of tumor cell receptors with specific ligands produced in certain host microenvironments. Rather than qualitative changes in tumor cell gene expression, these changes are probably reflected by quantitative changes in the expression of several genes that encode different classes of products important in each of the steps of brain metastasis.

Acknowledgment

Support from the National Foundation for Cancer Research (to G.L.N.) and the National Institutes of Health Training Grant (to D.G.M.) are gratefully acknowledged.

References

1. Liotta LA, Rao CN, Wewer UM: Biochemical interactions of tumor cells with the basement membrane. Annu Rev Biochem 55: 1037–1057, 1986
2. Nicolson GL: Cancer metastasis: tumor cell and host organ properties important in colonization of specific secondary sites. Biochim Biophys Acta 948: 175–224, 1988
3. Nicolson GL: Tumor and host molecules important in organ preference of metastasis. Semin Cancer Biol 2: 143–154, 1991
4. Nicolson GL: Paracrine and autocrine growth mechanisms in tumor metastasis to specific sites with particular emphasis on brain and lung metastasis. Cancer Metastasis Rev. 12: 325–343, 1993
5. Sugarbaker EV: Patterns of metastasis in human malignancies. Cancer Biol Rev 2: 235–278, 1983
6. Paget S: The distribution of secondary growths in cancer of the breast. Lancet 1: 571–573, 1889
7. Steck PA, Nicolson GL: Metastases to the central nervous system. In: Levine A, Schmidek H (eds) Molecular Genetics of Nervous System Tumors, pp 371–379, Wiley-Liss, New York, 1993
8. Einhorn LH, Burgess MA, Vallejos C, Bodey GP, Gutterman J, Mavligit G, Hersh EM, Luce JK, Frei E, Freireich EJ, Gottlieb JA: Prognostic correlations and response to treatment in advanced metastatic malignant melanoma. Cancer Res 34: 1995–2004, 1974
9. Patel JK, Didolkar MS, Pickren JW, Moore RH: Metastatic pattern of malignant melanoma. A study of 216 autopsy cases. Am J Surg 135: 807–810, 1987
10. de la Monte SM, Moore GW, Hutchin M: Patterned distribution of metastases from malignant melanoma in humans. Cancer Res 43: 3427–3432, 1983
11. Chason JL, Walker FB, Landers JW: Metastatic carcinoma of the central nervous system and dorsal root ganglia: a prospective autopsy study. Cancer 16: 781–787, 1963
12. Amer MH, Al-Sarraf M, Baker LH, Vaitkevicius VK: Malignant melanoma and central nervous system metastases: incidence, diagnosis, treatment and survival. Cancer 42: 660–668, 1978
13. Byrne TN, Cascino TL, Posner JB: Brain metastasis from melanoma. J Neuro-Oncol 1: 313–317, 1981
14. Brunson KE, Beattie G, Nicolson GL: Selection and altered properties of brain-colonizing melanoma. Nature 272: 543–545, 1978
15. Miner KM, Kawaguchi T, Uba GW, Nicolson GL: Clonal drift of cell surface, melanogenic, and experimental metastatic properties of *in vivo*-selected, brain-meninges-colonizing murine B16 melanoma. Cancer Res 42: 4631–4638, 1982

16. Kawaguchi T, Kawaguchi M, Dulski K, Nicolson GL: Cellular behavior of metastatic B16 melanoma in experimental blood-borne implantation and cerebral invasion. Invasion Metastasis 5: 16–30, 1985
17. Kawaguchi T, Kawaguchi M, Lembo T, Nicolson GL: Differential tumor growth of blood-borne B16 melanoma variants in cerebral dura mater is related to tumor-host reactions. Clin Exp Metastasis 7: 1–14, 1989
18. Schackert G, Fidler IJ: Site-specific metastasis of mouse melanomas and a fibrosarcoma in the brain or meninges of syngeneic animals. Cancer Res 48: 3478–3484, 1988
19. Schackert G, Price JE, Zhaang R, Bucana CD, Itoh K, Fidler IJ: Regional growth of different human melanomas as metastases in the brain of nude mice. Am J Pathol 136: 95–102, 1990
20. Ishikawa M, Fernandez B, Kerbel RS: Highly pigmented human melanoma variant which metastasizes widely in nude mice, including to skin and brain. Cancer Res 48: 4897–4903, 1988
21. Nicolson GL, Kawaguchi T, Kawaguchi M, Van Pelt C: Brain surface invasion and metastasis of murine malignant melanoma variants. J Neuro-Oncology 4: 209–218, 1987
22. Marchetti D, Menter D, Jin L, Nakajima M, Nicolson GL: Nerve growth factor effects on human and mouse melanoma cell invasion and heparanase production. Int J Cancer 55: 693–699, 1993
23. Belloni PN, Carney DH, Nicolson GL: Organ-derived endothelial cells exhibit differential responsiveness to thrombin and other growth factors. Microvasc Res 43: 20–45, 1992
24. Nakajima M, Irimura T, DiFerrante DT, DiFerrante N, Nicolson GL: Heparan sulfate degradation correlates with tumor invasive and metastatic properties of B16 melanoma sublines. Science 220: 611–613, 1983
25. Nakajima M, Irimura T, Di Ferrante N, Nicolson GL: Metastatic melanoma cell heparanase: characterization of heparan sulfate degradation fragments produced by B16 melanoma endoglucuronidase. J Biol Chem 259: 2283–2290, 1984
26. Barrett AJ, Kirschke H: Cathepsin B, cathepsin H, cathepsin L. Meth Enzyol 80: 535–561, 1981
27. Barrett AJ: Cathepsin D: purification of isoenzymes from human and chicken liver. Biochem J 117: 601–607, 1970
28. Nakajima M, Welch DR, Belloni PN, Nicolson GL: Degradation of basement membrane type IV collagen and lung subendothelial matrix by rat mammary adenocarcinoma cell clones of differing metastatic potentials. Cancer Res 47: 4869–4876, 1987
29. Albini A, Iwamoto Y, Kleinmann HK, Martin GR, Aaronson SA, Kozlowski JM, McEwan RN: A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. Cancer Res 47: 3239–3245, 1987
30. Herrmann JL, Menter DG, Hamada J-I, Marchetti D, Nakajima M, Nicolson GL: Mediation of NGF-stimulated extracellular matrix invasion by the human melanoma low-affinity p75 neurotrophin receptor: Melanoma p75 functions independent of *trkA*. Mol Biol Cell 4: 1205–216, 1993
31. Inoue T, Cavanaugh PG, Nicolson GL: Differences in transferrin response and numbers of transferrin receptors in rat and human mammary carcinoma lines of different metastatic potentials. J Cell Physiol 156: 212–217, 1993
32. Nicolson GL, Inoue T, Van Pelt CS, Cavanaugh PG: Differential expression of a $M_r \sim 90{,}000$ cell surface transferrin receptor-related glycoprotein on murine B16 metastatic melanoma sublines selected for enhanced brain or ovary colonization. Cancer Res 50: 515–520, 1990
33. Cavanaugh PG, Nicolson GL: Purification and characterization of a M_r 66,000 lung-derived paracrine growth factor that preferentially stimulates the *in vitro* proliferation of lung metastasizing tumor cells. J Cell Biochem 43: 127–138, 1990
34. Nicolson GL: Metastatic tumor cell attachment and invasion assay utilizing vascular endothelial cell monolayers. J Histochem Cytochem 30: 214–220, 1982
35. Auerbach RL, Alby L, Morrissey W, Joseph J: Expression of organ specific antigens on capillary endothelial cells. Microvasc Res 29: 401–411, 1985
36. Belloni PN, Nicolson GL: Differential expression of cell surface glycoproteins on various organ-derived microvascular endothelia and endothelial cell cultures. J Cell Physiol 136: 398–410, 1988
37. Hempstead BL, Martin ZD, Kaplan DR, Parada LF, Chao MV: High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. Nature 350: 678–683, 1991
38. Herlyn M, Kath R, Williams N, Valyi-Nagy I, Rodeck V: Growth regulatory factors for normal, premalignant and malignant human cells. Adv Cancer Res 54: 213–234, 1990
39. Nicolson GL: Cancer progression and growth: relationship of paracrine and autocrine growth mechanisms to organ preference of metastasis. Exp Cell Res 204: 171–180, 1993
40. Nicolson GL, Dulski KM: Organ specificity of metastatic tumor colonization is related to organ-selective growth properties of malignant cells. Int J Cancer 38: 289–294, 1986
41. Cavanaugh PG, Nicolson GL: Purification and some properties of a lung-derived growth factor that differentially stimulates the growth of tumor cell metastatic to the lung. Cancer Res 49: 3928–3933, 1989
42. Cavanaugh PG, Nicolson GL: Lung-derived growth factor that stimulates the growth of lung-metastasizing tumor cells: Identification as transferrin. J Cell Biochem 47: 261–271, 1991
43. Nicolson GL, Cavanaugh PG, Inoue T: Differential stimulation of the growth of lung-metastasizing tumor cells by lung (paracrine) growth factors: Identification of transferrin-like mitogens in lung tissue-conditioned medium. J Nat Cancer Inst Mgr 13: 153–161, 1992
44. Rodeck U, Becker D, Herlyn M: Basic fibroblast growth factor in human melanoma. Cancer Cells 3: 308–312, 1991
45. Nicolson GL: Tumor cell instability, diversification, and progression to the metastatic phenotype: From oncogene to oncofetal expression. Cancer Res 47: 1473–1487, 1987