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REVIEW

Brain-Metastatic Melanoma: a Neurotrophic Perspective

Dario MARCHETTI, Yvonne DENKINS, Jane REILAND, Andrea GREITER-WILKE, Jennifer GALJOUR, Brian MURRY, Jason BLUST, Madhuchhanda ROY

Department of Comparative Biomedical Sciences, Louisiana State University School of Veterinary Medicine, Baton Rouge, LA, U.S.A.

The brain is a unique microenvironment enclosed by the skull and maintaining a highly regulated vascular transport barrier. To metastasize to the brain, malignant tumor cells must attach to microvessel endothelial cells, invade the blood-brain barrier (BBB), and respond to brain survival and growth factors. Neurotrophins (NT) are important in brain invasion because they stimulate this process. In brain-metastatic melanoma cells, NT can promote invasion by enhancing the production of extracellular matrix-degradative enzymes such as heparanase, an enzyme capable of locally destroying both the extracellular matrix and the basement membrane of the BBB. We have examined human and murine melanoma cell lines exhibiting varying abilities to form brain metastases, and have found that they express low-affinity neurotrophin receptor $p75^{NTR}$ in relation to their brain-metastatic potentials. They do not, however, express *trkA*, the gene encoding the tyrosine kinase receptor TrkA, the high-affinity receptor for nerve growth factor (NGF), the prototypic NT. Presence of functional TrkC, the putative receptor for the invasion-promoting neurotrophin NT-3, was also expressed in these cells. Brain-metastatic melanoma cells can also produce autocrine factors and inhibitors that influence their growth, invasion, and survival in the brain. Synthesis of these factors may influence NT production by brain cells adjacent to the neoplastic invasion front, such as oligodendrocytes and astrocytes. In brain biopsies, we observed increased amounts of NGF and NT-3 in tumor-adjacent tissues at the invasion front of human melanoma tumors. Additionally, we found that astrocytes contribute to the brain-metastatic specificity of melanoma cells by producing NT-regulated heparanase. Trophic, autocrine, and paracrine growth factors may therefore determine whether metastatic cells can successfully invade, colonize, and grow in the central nervous system (CNS). (Pathology Oncology Research Vol 9, No 3, 147–158)

Keywords: brain metastasis, malignant melanoma, neurotrophins, neurotrophin receptors, $p75^{NTR}$, heparanase, astrocytes

Introduction

Brain metastasis, which occurs in 20-40% of all patients with cancer, is an important cause of cancer morbidity and mortality.[1] Surgical excision, radiation, and/or chemotherapy are clinically applied in patients with a resected primary tumor and single/multiple brain metastases, though none of the options has been designated as clinically preferential to others. Regardless of the treatment, the prognosis of patients with brain metastasis is grim.[1-3] The brain, because of its anatomical and physiological properties, provides a unique target for metastasis.[4] Homeostasis in the brain is highly sensitive to the slightest change in the local microenvironment due to its confinement by the skull. The brain is also surrounded by a formidable blood-brain barrier (BBB) that must be penetrated by brain-metastatic tumor cells, and it lacks the extensive lymphatic drainage which removes the fluid buildup that accompanies tumor growth in other parts of the body. To metastasize, tumor cells must complete a series of sequential and selective steps (*Figure 1*) resulting in subpopulations of cells with different angiogenic, invasive, and metastat-

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Correspondence: Dr. Dario MARCHETTI, Associate Professor, Department of Comparative Biomedical Sciences, Room 2522, School of Veterinary Medicine, Skip Bertman Drive, Louisiana State University-Baton Rouge, Baton Rouge, LA 70803, U.S.A., Phone: (225) 578-9897; Fax: (225) 578-9895
E-mail: dmarchetti@vetmed.lsu.edu

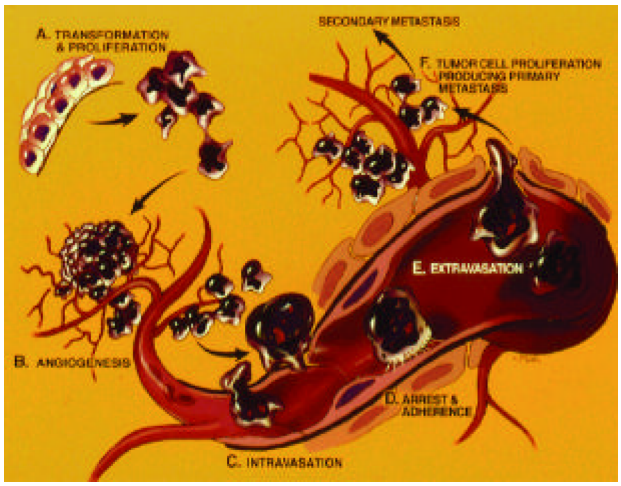

Figure 1. *Steps for metastasis. All metastatic cells that colonize distant organ sites such as the brain must first (A) proliferate, (B) respond to angiogenic events which feed tumor cells, (C) escape the primary tumor and enter the circulation, (D) circulate in the blood, arrest and adhere to the endothelium of the distant organ site, (E) exit from the microvasculature via extravasation processes, and (F) grow in response to the local microenvironment at the new site. Metastatic cells in the CNS must 1) adhere to brain microvessels, 2) penetrate the blood-brain endothelial barrier, and 3) grow in the brain.*

ic properties.[5] Studies have indicated that to produce brain metastasis, tumor cells must reach the vasculature of the brain, attach to microvessel endothelial cells, extravasate into the parenchyma, induce angiogenesis, and proliferate by responding to growth factors[6,7] (***Figure 1).***

CNS involvement is a common feature of metastatic melanoma, possibly due to a "homing" influence since melanocytes and neuronal subpopulations share a common embryologic origin (***Figure 2***). Malignant melanoma metastasizes to the brain with one of the highest frequencies of any cancer that is capable of colonizing the CNS. Patients with disseminated malignant melanoma frequently develop metastatic lesions in the brain and spinal cord that can result in severe and debilitating neurological complications.[2,3] Although melanoma metastasis formation in other organs may be tolerated or remain asymptomatic, once melanoma cells colonize the brain, tumor growth often results in a rapid decline in the quality of life and death ensues: almost 40% of melanoma patients will be treated for complications due to brain metastases. At autopsy, an additional 30-40% show CNS lesions.[2,3]

Malignant melanomas undergo progressive changes during their pathogenesis, especially those melanomas that progress to form brain metastases. Of the phenotypic changes that occur during metastatic melanoma progression, differences in the expression of receptors for paracrine growth factors and the production of various autocrine growth factors are important.[8,9] The significance of these autocrine factors in modulating the malignant properties exhibited by melanoma cells remains largely unknown, but they are thought to be important in allowing malignant cells to survive in unusual compartments such as the brain. NT are growth factors that promote neuronal cell survival, differentiation, and cell death.[10-14] The involvement of neurotrophins, their receptors, and neurotrophin-regulated heparanase in the development of brain metastasis are the subjects of this review.

Neurotrophins and Neurotrophin Receptors

Neurotrophins (NT) are a family of small (~13 kDa) proteins that are highly basic (P.I. 9-10.5). They are synthesized as prepropeptides, which are then N-terminally processed to proteins containing three interchain disulfide bonds.[10] The circulating forms of neurotrophins are non-glycosylated dimeric proteins of 26 kDa in size. Each protein monomer contains an elongated central axis made of an antiparallel β-sheet structure with a flattened hydrophobic face that is involved in dimer formation.[10] In addition to nerve growth factor (NGF), all members of the homologous NT family exhibit neurotrophic properties. Brain-derived neurotrophic factor,[15] isolated from brain tissue, shows significant amino acid homology (~50%) with NGF. Similarly, neurotrophin-3[16] isolated by various methods, neurotrophin-4 (NT-4) isolated originally from *Xenopus*, and its mammalian homolog neurotrophin-5 (NT-5) are all highly conserved in the amino acid sequence in the region of the central axis of the molecule.[10] The

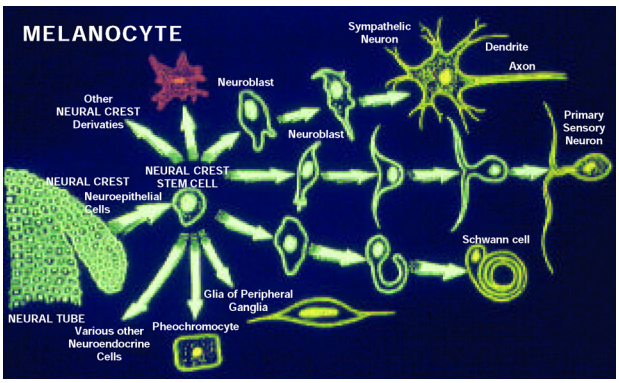

Figure 2. *Embryologic relationship between melanocytes and the most common neuronal cell populations, which are NT-responsive and possess specific cell-surface NTR. Examples include neurons of peripheral nervous system sensory and sympathetic ganglia, Schwann cells, glial cells and certain subpopulations of CNS cholinergic neurons. Another example of neural-crest derived, NT – responsive, are chromaffin cells: the chromaffin-derived pheochromocytoma cell line PC-12 is the most-studied cellular system to investigate the mechanism of action of NGF, the prototypic NT.*

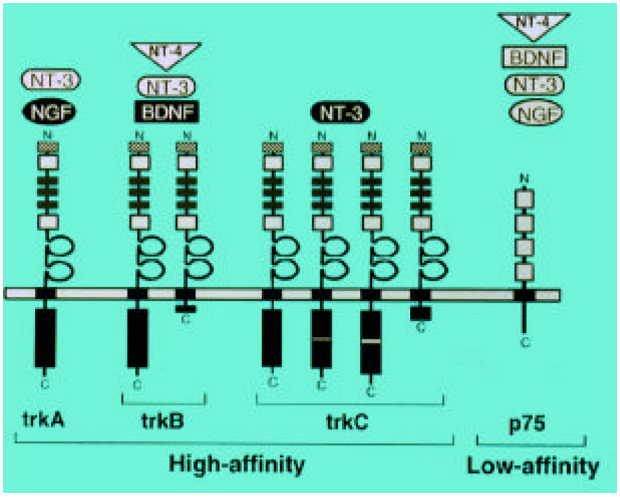

Figure 3. *Schematic representation of mammalian NT and the two different classes of neurotrophin receptors.[41] The primary NT ligand for each TRK receptor is indicated in the black boxes. Additional but secondary NT crossreactivities (NT-3 for TrkA as well as NT-3 and NT-4 for TrkB) are indicated in the white boxes. The p75^{NTR} binds all mammalian NT members (NGF, BDNF, NT-3, and NT4/5).*

recent discovery that NT precursor proteins and their proteolytically processed products may differentially activate pro- and anti-apoptotic cellular responses, through a preferential activation of NT receptors, promises to unveil yet another level of regulatory complexity.[11]

Neurotrophin receptors (NTR) have been historically divided[17] (although this designation is not entirely appropriate) into two affinity classes, a low-affinity receptor class ($K_D \sim 2 \times 10^{-9}$) and a high-affinity receptor class ($K_D \sim 2 \times 10^{-11}$; ***Figure 3***). The gene encoding the human low-affinity nerve growth factor receptor (NGFR or p75^{NTR}) was cloned by Chao and coworkers.[18] The human gene encodes a 75 kDa cell surface glycoprotein made up of 399 amino acids, including a 222 amino acid extracellular domain, a 22 amino acid transmembrane domain, and a 155 amino acid cytoplasmic segment. The molecule contains four cysteine-rich extracellular domains and a G protein-binding consensus sequence in the cytoplasmic domain.[18]

Studies originally established that the biological effects of NGF involve a tyrosine kinase activity.[19,20] Sequence analysis of p75^{NTR}, however, indicates that this molecule lacks a tyrosine kinase consensus sequence in the cytoplasmic domain.[18] Despite the absence of a tyrosine kinase domain, transfection of p75^{NTR} into non-neuronal cells enhanced tyrosine kinase phosphorylation following NGF stimulation.[21] The search for a high-affinity NGF receptor with tyrosine kinase activity resulted in the discovery of the TRK family of neurotrophin receptors.[22-25] The TRK family of tyrosine receptor protein kinases consists of several receptor molecules with varying degrees of specificity for the different members of the NT family. In addition to ***trkA***, hybridization cloning led to the discovery of the closely related proto-oncogenes ***trkB*** and ***trkC*** which constitute the high-affinity NT family members.[22-25] Each mature 140 kDa TrkA proto-oncogene protein contains a 375 amino acid extracellular domain, a 26 amino acid transmembrane domain, and a large cytoplasmic domain of 357 amino acids. The mature ***trkB*** and ***trkC*** proto-oncogenes encode molecules of 145 kDa which are known to also exist as truncated forms or to contain inserts in their tyrosine kinase domain ***(Figure 3)***. Each mature NTR cytoplasmic domain also possesses a tyrosine kinase consensus sequence that is followed by a highly conserved 15 amino acid post-kinase domain ***(Figure 3)***.

The TRK family members are widely distributed in neuronal tissues in addition to hematopoietic cells.[22-25] Of relevance, cellular responses to NT dimers are mediated by p75^{NTR} that binds all NT, as well as TrkA, TrkB, and TrkC receptors, each of which selectively binds only a subset of the neurotrophins: mainly, NGF binds TrkA, BDNF interacts with TrkB, while TrkC is the putative receptor for NT-3.[17,23]

Homozygous knock-out mice lacking NT or NTR have been very useful in elucidating the complex function of these developmental regulatory molecules.[14] There are major differences in the properties of knock-out mice that vary depending on which NT gene has been eliminated and the type of neuronal cell under observation.[14] For example, both NT-3 and its principal receptor, TrkC, regulate the proliferation and survival of neuronal precursors and the collateral branching of axons into target fields.[16,26-28] This is consistent with the lack of proprioceptor production in ***trkC*** (-/-) or NT-3 (-/-) mice.[29,30] In contrast, BDNF or ***trkB*** gene targeting seems to affect vestibular ganglia to the greatest degree,[15,31,32] whereas targeted disruption of NGF or ***trkA*** genes yields mice with defects in the superior cervical ganglia.[33-35] Importantly, gene targeting or knock-out experiments performed with each of the NT or their various receptors demonstrate profound effects on the survival of dorsal root ganglia neurons that have the same neuroectodermal origins as melanocytes (***Figure 2***). Although reports have not yet described an effect on melanocytes in these gene targeting studies, NT effects on melanocytes may be more complicated. There is evidence that many neuroectodermally-derived sensory neurons switch their neurotrophin dependence from BDNF or NT-3 during early embryonic development to NGF at later stages.[33] Therefore, the targeting of more than one NT or NTR in homologous recombination experiments can be used to observe an effect on melanocytes in null allele mice.

Since melanoma cells frequently exhibit genetic instability, a predisposition to switching expression of NTR genes to those most likely to support their survival in alternative tissue compartments is not unlikely. Developmental

changes in NT dependence parallels the progressive increase in $p75^{NTR}$ production that occurs during the progression of melanocytes to malignant melanoma. Additionally, phorbol 12-tetra decanoate 13-acetate (TPA) that was previously reported to induce $p75^{NTR}$ receptor production, has also been shown to induce synthesis of TRK receptors.[36-38] Interestingly, primary melanocyte cultures express low levels of *trkC* that are upregulated by TPA stimulation.[37] Although we did not find TrkA expression in malignant melanoma cells, we have observed high levels of both $p75^{NTR}$ and TrkC expression.[39,40] Importantly, presence of these NT receptors[41] in brain-metastatic melanoma resulted in enhancement of melanoma cell invasion and heparanase production.[42-45] Furthermore, we have recently reported that TrkC receptor functionality in these cells occurs via its association with a purine-analog sensitive kinase.[40]

We have formulated the hypothesis that brain metastases essentially represent a traumatic event related to brain-injury processes. Following mechanical/chemical brain insults increased NT/NTR presence is imperative in neuronal regeneration. These changes can be paralleled by brain invasive melanoma cells whose colonization within the brain microenvironment triggers NT production and their secretion by surrounding brain cells as response to the invasion event. Similarly, melanoma cells overexpressing NTR can benefit from such a synergistic microenvironment in terms of survival, growth, and further invasion into the brain parenchyma. NTR thus play important roles in melanoma progression to the brain while NT-regulated heparanase can be critical to this process.

Neurotrophin Receptor Signaling Mechanisms

The complexity of functional interactions between $p75^{NTR}$ and TRK receptors rivals that of other complex receptor systems.[17,46-49] It is generally agreed that tyrosine kinase receptors are involved in sequences of events that include ligand binding, leading to receptor dimer formation, and transactivation, resulting in tyrosine phosphorylation, with activation of serine/threonine phosphorylation cascades.[22-25,50,51] Active signaling complexes are frequently formed by interactions between receptor phosphotyrosines and proteins containing SH-2 (Src homology-2) tyrosine-binding domains.[52-63] Formation of this complex leads to tyrosine phosphorylation on Shc and the association of Shc with Grb2, another SH2-containing protein.[54,56-59,62] The association of Shc with Grb2 can lead to further complex formation with the $p21^{ras}$ nucleotide exchange factor Son of Seven-less-1 (SOS-1). This may result in increased GTP-binding and activation of $p21^{ras}$, a GTP-binding oncoprotein originally identified in a rat sarcoma virus.[61]

The downstream effectors of $p21^{ras}$ include proteins involved in serine/threonine phosphorylation cascades.[52] Studies have demonstrated that $p21^{ras}$ can coordinate the NGF-mediated, phosphorylation-dependent activation of several key growth and differentiation molecules[64] including: 1) c-Raf-1, a cytoplasmic ser/thr kinase discovered as the oncoprotein *v-raf* in a mouse sarcoma virus; 2) the mitogen-activated protein kinase/extracellular-signal-regulated kinase (MAPK/ERK kinase, MEK); and 3) the mitogen activated protein kinase (MAPK). The activation of MAPK can transiently induce the expression of a number of primary response genes that encode transcription factors, such as *c-fos*, *c-jun*, NGFI-B, and *krox24*.[53] The MAPK activity can also affect other ser/thr kinases and/or cytoskeletal elements.[63,64] MEK kinase (MEKK), a ser/thr kinase that can activate MEKs independently of c-Raf-1, has been observed to phosphorylate MEK in PC12 cells as they respond to NGF.[55,64]

Coexpression of $p75^{NTR}$ and TrkA resulted in increases in downstream signaling and neurotrophin responses including mitotic arrest, following neurite extension and neuronal maturation, relative to cells expressing only TrkA.[22,23,65,66] According to another model, $p75^{NTR}$ procures and presents bound NT molecules to members of the TRK receptor tyrosine kinase family to initiate signal transduction.[17,23] Little is known about $p75^{NTR}$ cooperative interactions with the other neurotrophin receptors, but evidence based on anti-$p75^{NTR}$ antibody injections into chick embryos suggests that BDNF and NT-3 may cooperate with $p75^{NTR}$ to form functional signaling pathways.[67] Collectively, these data emphasize the importance of cooperativity between the TRK family of receptors and $p75^{NTR}$ for enhancing NT capabilities of cells. Biochemical and functional interactions between TrkA and $p75^{NTR}$ have also been detected after co-immunoprecipitation and Western blotting.[41]

Dependent upon the cellular context in which it is expressed, $p75^{NTR}$ shows alternative functions. For example, in addition to receiving differentiation or survival signals in neuronal cells, $p75^{NTR}$ provide retrograde transport in certain neuronal cell types,[49] trigger apoptosis in virally transformed neuronal cells,[68] or promote survival when expressed in neutrophils.[69] The $p75^{NTR}$ cytoplasmic tail contains a 14 amino acid mastoparan-like domain.[18,70] Activation of a G-stimulatory protein complex in the presence (or absence) of NGF may lead to the production of cyclic AMP[71] by adenylate cyclase and activation of protein kinase A (PKA) followed by transcription factor activation.[71] Transfection studies involving sequence deletions in $p75^{NTR}$ of small segments in the cytoplasmic tail proved to be essential for high-affinity NGF binding involving TrkA in PC12 and NIH3T3 cells.[72,73] Certain properties of $p75^{NTR}$ also allow it to function in regulating survival and death of melanoma cells. In this regard, $p75^{NTR}$ is analogous to members of the tumor necrosis factor receptor (TNFR) superfamily, such as Fas (Apo I), TNFRI and II, and the B cell

antigen CD40, all of which regulate programmed cell death.[74,75] Therefore, it is apparent that $p75^{NTR}$ play bifunctional roles as a molecular switch that signals either cell survival or cell death dependant upon the cellular context.[76]

Importantly, a serine/threonine protein kinase that is sensitive to purine analogs and known as protein kinase N (PKN) has been isolated with $p75^{NTR}$ following NGF stimulation of PC12 cells.[77,78] The activation of this PKN in association with stimulation of ornithine decarboxylase activity play a potentially important role in the signaling pathways associated with $p75^{NTR}$.[50] Furthermore, we have recently demonstrated that there is an association between TrkC receptors and a purine-analog sensitive kinase in human brain-metastatic melanoma cells (70W).[40] We have also determined that this kinase is similar to the one known to associate with $p75^{NTR}$ and possesses an activity under the specific regulation by TrkC putative ligand, NT-3.[40] Therefore, purine-analogs sensitive kinases like PKN can represent a signaling component(s) common to NTR, playing roles in melanoma cell pathogenesis by generating constitutive downstream signaling. In relation to $p75^{NTR}$, the cooperative interaction of downstream signals from $p75^{NTR}$/PKN in amplifying signals pre-established by TRK may be important in brain-metastatic melanoma. In this case, when NT concentrations are high, the low-affinity activation of $p75^{NTR}$/PKN signals amplify the TRK response pathway. In contrast, when NT levels are low, $p75^{NTR}$ signals are driven along an alternate pathway, allowing $p75^{NTR}$ to act as a sensitive molecular switch due to its low-affinity for NT.

Activation of the sphyngomyelin cycle serves as an alternate signaling pathway for $p75^{NTR}$.[79] The sphyngomyelin pathway also seems to be important during signaling by TNF receptors ($p75^{NTR}$ is a member of the TNF receptor superfamily), and this pathway appears to involve a ceramide-activated protein phosphatase.[80] This alternate form of signal transduction by $p75^{NTR}$ may be important to cells invading the brain. Brain tissue injured by tumor cell invasion can provide a ready source of ceramide that might also influence invading cells.

Neurotrophin Receptors and Progression of Malignant Melanoma Cells

During malignant progression, melanoma cells show progression-associated increases in the expression of $p75^{NTR}$,[6,9] as witnessed by *in situ* examination of $p75^{NTR}$ levels in advanced stages of malignant melanoma.[81] Human melanoma cells established in short-term tissue culture from brain metastases exhibit characteristic chromosomal alterations.[82] Importantly, although $p75^{NTR}$ expression was not examined in these cells[82], the gene is located at 17q21-22 and may be amplified in tumor cells containing the isochromosome.

We have examined the role of NTR in brain invasion and colonization of malignant melanomas. Using a human melanoma variant cell subline (70W) that has the capacity to form brain colonies in nude mice, we have studied the effects of neurotrophins and growth factors on their malignant properties. The 70W subline was derived as one of the series of human MeWo melanoma cell variants selected by treatment with wheat germ agglutinin.[83] Parental MeWo cells exhibit intermediate metastatic potential compared to other cell lines, such as the nonmetastatic 3S5 and the brain-metastatic 70W cell variants. Of note, 3S5 and 70W cells possess opposite metastatic capabilities when injected *in vivo* in nude mice: while 3S5 are nonmetastatic, 70W are highly aggressive and brain-metastatic,[84] being the first reported example of human melanoma cells capable of metastasizing to the brain when injected intravenously in nude mice.[83] Furthermore, target organ site colonization by the 70W line is similar to the clinical presentation of melanoma metastasis. Using the MeWo melanoma cellular system (MeWo parental, 3S5 and 70W variants), we have shown that overexpression of $p75^{NTR}$ is associated with brain colonization, enhancement of extracellular matrix invasion,[39,84] and heparanase activity.[84]

Neurotrophins Enhance Invasion and Heparanase Production in Brain-metastatic Melanoma Cells

During metastasis formation, migrating tumor cells are confronted by natural tissue barriers, such as basement membranes (BM) that surround the blood vessels[85,86] or extracellular matrix (ECM) that is an integral part of the BBB. The ability of malignant cells to penetrate these barriers depends upon the production and activation of enzymes capable of ECM degradation.[87-89] ECM/BM are rigid structures formed from such macromolecules as type IV collagen, laminin, entactin, nidogen, fibronectin, and proteoglycans,[90] one type being heparan sulfate proteoglycans or HSPGs. It is known that HSPGs play a central role in embryonic morphogenesis, angiogenesis, neurite outgrowth, and tissue repair.[91-94] ECM/BM HSPGs also provide a readily available storage depot for growth factors and cytokines.[95] Since HSPGs are now recognized as active biological modulators, their degradation at the level of HS chains is expected to have significant regulatory consequences in cancer metastasis.[91] Indeed, HSPG catabolism is observed in inflammation, wound repair, diabetes, and neoplasia, including melanoma.[96,97] Melanoma heparanase responsible for HS degradation cleaves HS at specific intrachain sites resulting in the formation of fragments of discrete molecular weight.[96,98] Therefore, heparanase was identified as a member of the family of endo-β D-glucuronidases.[97,99] Importantly, heparanase differs from heparinases or other HS-specific elimination enzymes (heparatinases) by cleaving HS into characteristic distinct molecular weight

fragments of approximately 10-20 sugar units in size.[96] Heparanase activities have also been described in the immune system and in cancer cells other than melanoma.[95,97,100] Increased levels of heparanase activity are associated with metastatic melanoma and other invasive tumors types, and copious evidence has demonstrated its role in tumor cell invasion into distant organs.[95,97]

We have made the following observations: 1) highly brain invasive human melanoma cells degrade purified ECM-HS and HS cell-surface subpopulations faster than sublines of lower metastatic potential,[42,84] 2) heparanase is responsible for this HS degradation,[42,96] 3) in direct correlation with both increased invasiveness and presence of their specific NTR, select NT members augment heparanase production in brain-metastatic melanoma, making it a major candidate enzyme responsible for ECM degradation;[42,44,84] and 4) heparanase recognizes specific motifs within HS chains associated with both the binding domains to angiogenic factors and to an HS-interacting protein (HIP), recently cloned and characterized.[96]

Of note, human heparanase has not been purified nor well-characterized or cloned until 1999.[100-103] Therefore, molecular tools to explore the potentially important roles of heparanase in disease have been lacking for almost 25 years, following the first reports describing its enzymatic activity. Interestingly, the newly discovered cDNA sequences of human heparanase derived from normal and tumor cells represent the same gene.[100-103]

We have postulated that heparanase plays two critical roles in the biology of brain metastasis, which are 1) in local invasive processes by degrading the HS chains of HSPGs, and 2) in the release of HS-bound angiogenic factors at the metastatic site with the brain as the ideal environment due to its high levels of NT production. Heparanase can therefore be dually relevant in brain-metastatic melanoma in consideration of the strong angiogenic properties exhibited by melanoma cells in the brain. Although metastasizing cancer cells may produce as many as 28 different matrix-digesting proteases, the new findings show that there is only one heparanase. Heparanase inhibition not only inhibits cancer cells' ability to invade, but also hinders the formation of new blood vessels that feed tumors (angiogenesis).[104] Another important aspect in the area of brain invasion and metastasis is therefore investigations aiming to assess heparanase contributions to angiogenic events. ECM HSPGs serve as a reservoir for angiogenic factors, like basic fibroblast growth factor (bFGF), that can be extracted from subendothelial ECM produced *in vitro*.[93,105] Displacement of bFGF from the ECM by heparanase can provide a novel mechanism for induction of neovascularization in normal and pathological conditions.[106] Several studies have indicated that heparin and HS inhibit the mitogenic activity of angiogenic bFGF and at the same time stabilize and protect the molecule from inactivation.[107] bFGF is stored in ECM in a highly stable, inactive form. Its release from ECM as a complex with HS fragments can result in a form of bFGF that is more stable than free bFGF and capable of binding the high-affinity plasma-membrane receptors.

We have evaluated the ability of human melanoma heparanase, purified through chromatographic techniques or prepared as recombinant protein, to modulate bFGF activity as controlled release from HSPGs by observing *in vivo* angiogenesis induction.[108] Secondly, we have found that a group of suramin analogs promoted nearly complete inhibition of heparanase-induced invasion and angiogenesis.[108] These results further emphasize the importance of heparanase in brain invasive and angiogenic mechanisms,[95] and the potential clinical application of heparanase inhibitors such as suramin analogs or others (i.e. low-molecular weight inhibitors) in angiogenic-dependent cancers like brain-metastatic melanoma.

Brain Tissue Neurotrophin Production at the Melanoma Invasion Front

After establishing that 70W melanoma cells produce growth factors, including transforming growth factors α/β (TGF-α/β), bFGF, and NGF-regulating interleukin-1 (IL-1β we reasoned that these factors might act as paracrine factors influencing NT production in the brain. Many of these factors can stimulate brain astrocytes or oligodendrocytes to produce NT. Therefore, we examined whether brain-invading melanoma cells induce changes in NT concentration or distribution at the brain invading edge of melanoma tumors *in vivo*. Brain-tissue samples from human melanoma metastases and uninvolved brain tissues in adjacent sections were examined immunohistochemically for the presence of NT. Staining of serial sections with anti-NT monoclonal antibodies revealed increased concentrations of NT (in particular NGF and NT-3) in the tumor-adjacent tissue at the invasive front. Staining was highest at the interface between the melanoma tumor and adjacent normal brain tissue and gradually decreased in concentration until NT was undetectable at more distant sites.[110] Controls without primary antibody[110] or uninvolved brain tissue progressively distant from the melanoma lesion[110] possessed very low or undetectable levels of NT using these methodologies.

Astrocytes Contribute to the Brain-metastatic Specificity of Melanoma Cells by Producing Heparanase

Astrocytes, which are among the first brain cells encountered by extravasating melanoma cells, produce NT.[111] Furthermore, they are capable of binding NT since expressing members of the TRK receptor family and $p75^{NTR;\ 111}$ and are relevant because astrogliosis can be at

times a pathologic trauma in response to brain invasive events: injury-reacting astrocytes are frequently found in areas surrounding melanotic lesions. Therefore, astrocytes can play important roles in the development of brain metastases. To test this hypothesis, we employed purified *in vitro* astrocytic cultures and investigated the presence of heparanase. Primary glial cells were obtained from newborn rat and mouse cerebra using established purification methods.[112] Their identification as astrocytes was confirmed by positive immunoreactivity with an antibody against the astrocyte-specific intermediate filament glial fibrillary acidic protein (GFAP).[112] We next examined the astrocyte cultures for expression of heparanase. A specific heparanase transcript was detected by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR).This transcript was upregulated 3-fold in astrocytes previously incubated with purified and biologically active NGF.[113] Similar results were obtained using human brain-metastatic 70W cells. Heparanase activity was also detectable and NGF-regulated in cellular extracts from both purified astrocytes and brain-metastatic cells. This was shown by the appearance of distinct HS degradation products detected in agarose gel shift assays or by high-speed gel permeation column chromatography (HPLC analysis).[113]

We also analyzed heparanase activity in brain-metastatic melanoma cells and astrocytic cell populations in logarithmic growth by obtaining HPLC-derived elution profiles of HS digested products at various incubation times.[84] Cultures of highly brain-metastatic 70W cells showed a gradual and time-dependent increase in heparanase activity for up to 72 hr. After this time interval, the levels of heparanase in the cultures plateaued. Interestingly, cultures of astrocytes also produced heparanase in a time-dependent manner. Moreover, coincubation of brain-metastatic melanoma cells and astrocytes in equicellular numbers resulted in a super-additive increase of enzymatic activity above that expected of both cell types.

To determine if poorly metastatic melanoma cells acquire an increased metastatic potential following heparanase gene upregulation, we constructed eukaryotic expression vectors that contained the full-length human heparanase cDNA and used them to transfect melanoma cells. Transfection of the human heparanase gene into cells of low-invasive potential and heparanase content resulted in functional enzymatic activity and in significantly increased (7- to 14-fold) invasion of transfected cells using *in vitro* chemoinvasion assays with purified HSPG as barrier.[114]

Finally, we incubated brain-metastatic melanoma cells with astrocyte-conditioned medium (ACM)[34] and examined its effects on their invasive characteristics. Consistent increases in *in vitro* invasion were found following exposure of these cells to ACM. Invasion was most pronounced using ACM from NGF-treated astrocytes, and the invasion effects of ACM were completely abrogated in the presence of heparanase antibodies.[114] The invasion enhancement caused by this NGF treatment was also abolished in presence of a neutralizing NGF monoclonal antibody, confirming the relevance of melanoma/astrocyte heparanase and its NT-regulation to brain invasion events.

The Brain as a Unique Compartment for the Invasion and Growth of Malignant Melanoma Cells

Homeostasis and the control of material flow into the brain is strictly regulated by the BBB. Anatomically, the barrier is defined by specialized endothelial cells that are joined by an extensive network of tight junctions. The endothelial barrier is supported by a thick basement membrane and an underlying layer of astrocytes that control the traffic of ions, nutrients, and cells into the brain. Brain-metastatic cells must breach this formidable barrier to invade and colonize the brain parenchyma. As discussed above, invasion of the brain requires that metastatic cells increase their expression of certain cell surface receptors (NTR), degradative enzymes (heparanase), growth factors and cytokines (TGFα/β, bFGF, IL-1β and others). They must also respond to invasion-stimulating cytokines such as NT and paracrine growth factors.

Brain-metastasizing melanoma cells express relatively high levels of basement membrane hydrolytic enzymes, such as type IV collagenases, cathepsins, plasminogen activators, and of relevance, heparanase. Although highly metastatic cells generally expressed higher amounts of degradative enzymes than nonmetastatic cells, some of these enzymes are induced to even higher levels by the microenvironment,[6] or are provided by certain normal cells (microvessel endothelial cells and astrocytes, among others).[6,45] If the appropriate paracrine signals are received by malignant cells, they can be stimulated to increase the synthesis and release of BBB-degrading enzymes. For example, as discussed in previous sections, we found that brain-metastatic human and murine melanoma cells are sensitive to exogenous NGF, and treatment of brain-metastatic cells with NGF increases their expression of type IV collagenase (gelatinase A)[39] and, importantly, heparanase.[42,43,84]

Cellular Responses to Brain Tissue Injury as a Paradigm for Brain Metastasis

Astroglial cells constitute the primary cellular response following brain injury.[115] Astrocytes are the predominant cell type in the brain outnumbering neurons by a factor of ten to one. By numbers, astrocytes make up one-third of the cerebral cortex; however, as a population of cells they are widely heterogenous.[116,117] One of the earliest patho-

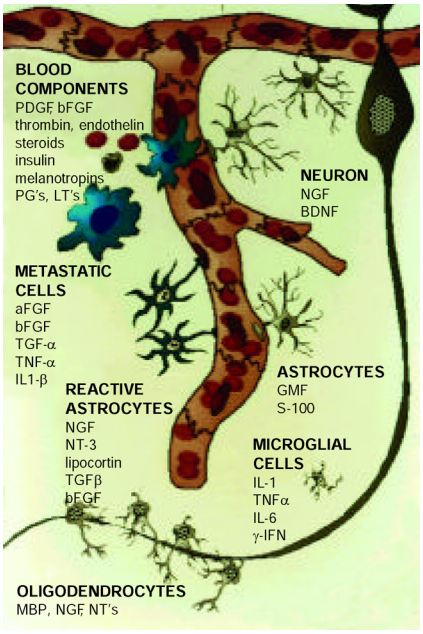

Figure 4. *Reciprocal interactions between brain-invading melanoma cells and normal cells in the brain microenvironment. Tumor cells release cytokines that can affect host cells, such as parenchymal cells, endothelial and glial cells, astrocytes and brain tissue extracellular matrix (ECM). Reactive astrocytes can arise from stimulation by factors released by invading melanoma cells. In turn, brain cells can release factors that stimulate tumor cell motility and invasion. Astrocytes, oligodendrocytes, and neurons can release NT and ECM degradative enzymes (i.e., heparanase produced by astrocytes)[108] in response to brain-invading melanoma. Conversely, these cells secrete growth factors and cytokines which can synergistically regulate NT synthesis and activity in normal brain cells.*

logical responses to brain trauma involves astrocyte swelling occurring predominantly in the perivascular astrocytic endings.[118,119] In experimental brain tumors, cerebral edema has been associated with significant alterations in vascular permeability.[120] If the BBB is compromised, astrocyte swelling may involve vasogenic edema. In this case, the astrocytes swell as they take-up proteins and water that may become cytotoxic due to increase potassium and glutamate.[115,118,121] It is generally believed that astrocyte swelling is caused by increases in intracellular osmolarity followed by water influx. This can occur without loss of BBB integrity and perhaps simply represents a redistribution of water from the neuronal cell compartment to the astrocytic cell compartment. This mild form of astrocyte swelling is generally not as severe as the astrocyte swelling that can result from vasogenic edema associated with the trauma caused by tumor cell invasion. If astrocyte swelling becomes too severe it can cause astroglial cells to depolarize, leading to the loss of homeostatic ion gradients and membrane rupture resulting in cell death. These dynamic astrocyte changes in response to tumor cell invasion can lead to increased intracranial pressure and further complications. This tumor-induced response by astrocytes is being investigated as a cause, or one of the causes, of brain metastases generating severe symptoms, such as paralysis, headache, seizures, and impaired cognition.

Vasogenic edema leads to the influx of thrombin platelet derived growth factor, steroids, insulin, and various cytokines from the blood and lymphocytes as well as endothelin, ATP and bFGF from endothelial cells (***Figure 4***). The induction of reactive astrocytes, when associated with tumor cell invasion, is likely triggered by endogenous factors in the brain in addition to those provided by the invading tumor cells (***Figure 4***). We have observed reactive gliosis in brain tissue associated with the melanoma invasion front, illustrating the cellular response of the adjacent brain tissue.[42] In addition to morphological changes, the adjoining brain cells produce high levels of NT (NGF, NT-3) in comparison to uninvolved brain tissue.[110] Thus, brain-metastatic melanoma cells may induce the production of brain cytokines such as NT that aid in the survival and invasion of melanoma cells in the CNS.

Concluding Remarks and Perspectives

Clearly, we have much more to learn about the mechanisms utilized by melanoma cells in colonizing the brain. In this review we have attempted to identify certain topics that will require further examination. For example, one future goal is to test in ***in vivo*** settings the hypothesis that $p75^{NTR}$ overexpression and NT responsiveness are truly determinants for invasion and establishment of clonal dominance by human melanoma cells metastatic to the brain. Our laboratory has selected both high- and low-$p75^{NTR}$ ($p75^{NTR\text{-}H/L}$) variants by fluorescence-activated cell sorting (FACS) and related ***in vitro*** invasion and NT responsiveness to $p75^{NTR}$ content.[122] By also using $p75^{NTR\text{-}H/L}$ subclones obtained from transfection studies, we will investigate the extent of $p75^{NTR\text{-}H/L}$ expressors to colonize the brains of nude mice. We will also use a dominant negative strategy that will allow interference with $p75^{NTR}$ – mediated signaling after infection of melanoma cells with recombinant adenovirus containing a $p75^{NTR}$ dominant negative mutant. Brain tumor growth by dominant negative inhibition of $p75^{NTR}$ will be determined by injecting the viral-infected cells in nude mice. These investigations are of significance because they will provide a rigorous and unequivocal proof of $p75^{NTR}$ as a key determinant responsible for brain metastases formation. Lack of such knowledge represents a problem, because acquiring the ability to therapeutically inhibit brain-metastases through $p75^{NTR}$ – mediated mechanisms is unlikely without it.

Another future aim includes the complete characterization of NT-regulated heparanase gene expression in melanoma progression and mechanistic studies (*in vitro* as well as *in vivo*) to clarify its involvement in both invasive and angiogenic processes. Now, that the heparanase gene has been cloned and its cDNA sequence is known, more complete strategies (i.e., the development of heparanase gene knock-outs, the application of sense/antisense, small interference RNA, or other technologies) can be formulated to provide direct evidence of the roles of heparanase in brain metastasis.

Equally compelling, questions related to the potential benefit of the therapeutic suppression of heparanase in brain-metastatic events remain unanswered. Additional studies with normal brain and brain-metastatic cells, as well as *in vitro* and *in vivo* experiments using specific inhibitors of heparanase or amplifying the use of sense/antisense strategies will be useful to address these questions.

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