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FAST TRACK

Heparanase Mechanisms of Melanoma Metastasis to the Brain: Development and use of a Brain Slice Model

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Abstract Heparanase (HPSE-1) is an endo-β-D-glucuronidase that cleaves heparan sulfate (HS) chains of proteoglycans (HSPG), and its expression has been associated with increased cell growth, invasion, and angiogenesis of tumors as well as with embryogenesis and tissue development. Since metastatic cancer cells express HPSE-1, we have developed an orthotopic brain slice model to study HPSE-1 involvement in brain-metastatic melanoma. This model allows for the characterization of tumor cell invasion at both quantitative and qualitative levels. Brain-metastatic melanoma cells (B16B15b) showed augmenting levels of HPSE-1 protein expression in a time-dependent manner. Secondly, B16B15b cells pre-treated with HPSE-1 showed a significant increase in the number of cells that invaded into the brain tissue. Finally, HPSE-1 exposure-augmented invasion depth in brain sections by brain-metastatic melanoma cells. We concluded that applying this brain slice model can be beneficial to investigate HPSE-1- related in vivo modalities in brain-metastatic melanoma and brain invasion in general. These results also further emphasize the potential relevance of using this model to design therapies for controlling this type of cancer by blocking HPSE-1 functionality. J. Cell. Biochem. 97: 217–225, 2006.

Key words: heparanase; brain metastasis; brain slice model; invasion; melanoma

Mechanisms responsible for the progression of malignant melanoma to highly aggressive brain-metastatic disease remain largely unknown. Brain-metastatic melanoma and elevated levels of heparanase (HPSE-1) are known to be correlated [Marchetti et al., 1993, 1996; Murry et al., 2005]. HPSE-1 is a endo-β-D-glucuronidase that is involved in degrading the extracellular matrix (ECM) by specific cleavage of heparan sulfate chains (HS) present on HS proteoglycans (HSPG) [Marchetti et al., 1993, 1996; Marchetti and Nicolson, 2001]. When HPSE-1 degrades HSPG, it releases protein modulators that are involved in important cellular functions like adhesion, inflammation, migration, angiogenesis, embryogenesis, and metastatic invasion [Parish et al., 1987; Nakajima et al., 1988; Vlodavsky et al., 1991; Hulett et al., 1999; Kussie et al., 1999; Toyoshima and Nakajima, 1999; Vlodavsky et al., 1999; Vlodavsky and Friedmann, 2001; Zetser et al., 2003; Sasaki et al., 2004]. Elevated HPSE-1 levels have been shown to correlate with increased aggressiveness of metastatic tumors. In addition, it is known that patients with metastatic disease show augmented HPSE-1 levels in their sera and urine [Nakajima et al., 1988; Kelly et al., 2003], and post-operative patients with high HPSE-1 mRNA content have a reduced chance for survival [Vlodavsky et al., 1999; Vlodavsky and Friedmann, 2001].

The orthotopic brain slice model is a method where cultures of nervous tissue can be maintained at the interface between air and culture medium [Stoppini et al., 1991; Ohnishi et al., 1998; Matsumura et al., 2000]. It represents an

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attractive system because these cell cultures maintain their cytoarchitecture, for example, cortical lamination and presence of pyramidal cells. Secondly, they retain the biochemical and electrophysiological properties of neural cells for up to 1 month in culture. This model also makes possible investigating molecular events which normally only occur in vivo outside the living body [Ohnishi et al., 1998; Matsumura et al., 2000]. Finally, this brain slice model allows for the ability to monitor tumor invasion at both quantitative and qualitative levels. The objective of our studies was to use this model to assess effects that HPSE-1 has on the invasive properties of brain-metastatic melanoma cells while being able to monitor cells in in vivo-like settings. In the present report, we provide first-time evidence that brain-metastatic melanoma cells (B16B15b) plated on these organotypic cultures showed a time-dependent differential HPSE-1 expression. Additionally, they possessed a significant increase in in vivo invasion when they were pre-treated with HPSE-1 in a dose-dependent manner.

MATERIALS AND METHODS

Cell Lines and Tissue Culture

Early-passage murine brain-metastatic melanoma B16B15b and non-brain metastatic B16F10 cells [Fidler, 1973; Marchetti et al., 1993, 1996] were maintained as monolayer cultures in a 1:1 (v/v) mixture of Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS) (v/v) and in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 μg/ml of blasticidin, respectively. Cells were maintained at 37°C in a humidified 5% CO_2/95% air (v/v) atmosphere and passaged using ethylenediaminetetraaceticacid (EDTA) [2 mM in phosphate buffer saline (PBS)] before reaching confluence.

Isolation of B16B15b/B16F10 Clones That Constitutively Express the Enhanced Green Fluorescent Protein (EGFP)

The *EGFP* gene was PCR-amplified from pTF9200 [Foster et al., 1998] and ligated into pEFα-TA TOPO (Invitrogen Corporation, Carlsbad, CA), resulting in plasmid pEFα-EGFP, which specifies the EGFP gene under the constitutive EFα promoter. Subconfluent B16B15b and B16F10 cells were transfected in 6-well plates with linearized pEFα-EGFP using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Cell lines that stably expressed EGFP were selected in growth medium containing blasticidin (10 μg/ml) by limiting dilution, clonal isolation, and expansion, as described previously [Foster et al., 1999]. Twenty-five clones were isolated, and the stable expression of EGFP was monitored by fluorescence microscopy. Specifically, four individual B16B15b clones, numbered sequentially and designated B16B15b-EGFP, expressing high levels of EGFP, were isolated. They exhibited cell morphology and growth characteristics similar to parental B16B15b cells. Clone B16B15b-EGFP No. 2 was utilized in described studies. B16F10 clones were produced by the same method and clones expressing high EGFP levels were selected for our experiments.

HPSE-1 Source and Activity

Recombinant HPSE-1 was used and purified as previously described [McKenzie et al., 2003]. Briefly, Sf9 insect cells, transfected with baculovirus transfer vectors containing HPSE-1 subunits, were grown in SF900II serum-free medium (Gibco BRL, Grand Island, NY) for high-titer stocks. Tni cells cultured in suspension using ExCell405 serum-free medium (JRH Bioscience, Lenexa, KS) were infected with high-titer stock for 48 h, and cells were subsequently removed by centrifugation. The supernatant was then tested for HPSE-1 activity, filtered through a 0.45 μm filter, and loaded on a HiTrap heparin column (Amersham Biosciences, Piscataway, NJ). The column was subsequently washed in wash buffer (150 mM NaCl, 25 mM Tris-HCl, pH 7.5) then eluted using a 100 ml gradient of 0.15–1.0 M NaCl in 25 mM Tris-HCl, pH 7.5. Collected fractions (1 ml) were screened for HPSE-1 activity (Heparan sulfate degrading enzyme assay kit; Takara Mirus, Madison, WI). HPSE-1 eluted at 0.67 M NaCl, as expected [McKenzie et al., 2003]. Dose-dependent HPSE-1 activity was determined by the heparan sulfate assay kit as previously described [Reiland et al., 2004].

Brain Slice Model

Brain tissues used for the organotypic brain slice model were obtained from 6 to 8 week old female C57BL/6 mice (Harlan Sprague Dawley, Indianapolis, IN). Prior to euthanasia, mice were quarantined for 1 week, housed in a

barrier facility, and fed Purine Lab Chow 5001. Mice were first anesthetized with isoflurane and killed by decapitation. The mouse head was then plunged into 1% povidone-iodine solution. The brain was subsequently extracted from the skull and immediately submerged in ice-cold sterile PBS. Next, the anterior portion of the brain was mounted and imbedded in a 3% agarose column with the anterior portion of the brain facing upward. Finally, coronal brain sections (500 μm thick) were obtained using a vibratome (Vibratome, St. Louis, MO). Brain sections were then transferred onto the polycarbonate membrane with a pore size (0.4 μm) in the upper chamber of a transwell tissue insert in a six-well plate (Corning, New York, NY), and 2 ml of Dulbecco's modified Eagle's/Ham's F-12 medium (DMEM/F12) medium (Sigma, St. Louis, MO) supplemented with 10% FBS, and 100 U/ml of penicillin/streptomycin were added to bottom wells of six-well plates. After allowing the brain to equilibrate for 24 h, B16B15b/B16F10-EGFP cell suspensions ($1.0–4.0 \times 10^5$ cells) were plated on the surface of the caudate nucleus and incubated for different amounts of time.

Brain Slice Neuronal Viability Assays

Neuronal viability in brain slices was assessed in terms of cellular uptake of propidium iodide (PI) (Sigma) before and after treatment with N-methyl-D-aspartic acid (NMDA) (Sigma). Morphological examinations were performed periodically on sections that had been incubated for longer than 72 h. Brain slices were incubated with PI, after a 15 min exposure to 100 μM NMDA, for 1 h or 24 h, respectively. PI signals were viewed using a TRITC filter under a Axioplan fluorescence microscope (Carl Zeiss Microimaging, Thornwood, NY). PI was dissolved in a serum-free solution containing modified Eagle's medium (MEM; 75%), PBS (25%), 2 mM L-glutamine, and 6.5 mg/ml glucose to a final concentration of 4.6 μg/ml, as previously reported [Ohnishi et al., 1998; Matsumura et al., 2000].

Brain Slice Model Invasion Assays

For invasion assays using the orthotopic brain slice model, melanoma-EGFP cells were suspended in Dulbecco's modified Eagle's medium (DMEM/F-12) supplemented with 10% FBS, and with 100 U/ml of penicillin and streptomycin at a concentration of 2.0×10^5 cells/μl. Directly before implanting melanoma cells on brain slices, purified recombinant HPSE-1 was added to the cell suspension at several concentrations (0, 10, 25, and 50 ng/ml, respectively).

Next, cells ($1.0–4.0 \times 10^5$ in 0.5 μl) were implanted superficially on the caudate putamen of each brain slice (three brain slices/treatment group/6-well plate) and incubated for 72 h at 37°C. As a negative control, melanoma-EGFP cells were suspended in the same medium previously mentioned and implanted on the brain slice without any treatment.

Serial sections were taken from brain slices that had been implanted with HPSE-1- treated melanoma cells (brain-metastatic B16B15b-EGFP or non brain-metastatic B16F10-EGFP) and with corresponding non HPSE-1- treated control cells. Immunohistochemistry (IHC) was performed on these serial sections with anti-green fluorescence protein (GFP) polyclonal antibody in order to definitively mark melanoma-EGFP cells that had invaded into the brain slice tissue. Analysis of serial sections was performed blindly with a representative tissue sample. Invasion was measured in two different ways. First, melanoma cell invasion was assessed as the number of cells invading beyond the surface of the brain per unit of area (12 μm^2). Second, a method was used to determine invasion by measuring the depth of the most invasive cells per brain slice. The same counting area was used for all sample measurements followed by statistical analysis.

Brain Slice Model Time Course Assays

HPSE-1 expression patterns of malignant melanomas were monitored at different invasion points by performing time-course assays. Using the orthotopic brain slice model, B16B15b-EGFP cells were suspended in medium and plated as previously mentioned. Melanoma cells were implanted without any additional treatment, and cells were incubated for 2, 12, and 24 h, respectively.

Tissue Preservation

After B16B15b-EGFP cells and the brain slice were allowed to incubate, tissue sections were rinsed in sterile PBS and then fixed in formalin for 24 h. Following formalin fixation, brain slices were rinsed in sterile PBS and then incubated for 24 h in 30% sucrose in PBS [Parlato et al., 2002]. The tissue was rinsed with PBS again and flash frozen in isopentane

over dry ice. The frozen sections were embedded in optimal cutting temperature medium (OCT), mounted on a cryomold, and 10 μm-thick serial sections were taken from a superior view of the 500 μm brain slices cut at −28°C [Emmert-Buck et al., 1996; Sugiyama et al., 2002].

Immunohistochemistry

Immunohistochemical staining was performed using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to instructions provided by the manufacturer. Briefly, formalin-fixed, OCT-embedded tissue sections were mounted on poly-L-lysine coated silanized slides. Endogenous peroxidase was blocked by incubating the sections in 3.0% hydrogen peroxide. After blocking non-specific reactivity with rabbit serum for 30 min at room temperature (25°C), sections were incubated at 4°C for 60 min with the anti-rabbit polyclonal GFP antibody raised against all variants of recombinant Aequorea GFP (Novus Biological, Littleton, CO) or with anti-human HPSE-1 rabbit polyclonal antibody raised against human HPSE-1 [Fairbanks et al., 1999]. Following rinsing, slides were incubated with biotinylated anti-rabbit IgG and then with Vectastain ABC reagent (Vector). Peroxidase activity was determined using NovaRED substrate kit (Vector), and slides were counterstained with Mayer's hematoxylin. As a negative control, sections were subjected to normal serum blocking omitting the primary antibody.

Statistical Analyses

All statistical analyses were conducted by using the SAS® statistical package (version 9.1.2) (SAS Institute, Cary, NC). To determine if there was a correlation between the number of cells invading and cell invasion depth, the Pearson correlation coefficient was calculated and significance was tested. In order to establish if this correlation could be used as a predictive model for overall cell invasiveness, a regression analysis by treatment group was also performed. Finally, analysis of covariance was carried out studying heterogeneity of the slopes with cells as a covariate in order to conclude if there was a difference in invasion between treatment groups. The results are expressed as mean ± standard error of the mean. All tests were considered significant at $P < 0.05$.

RESULTS

Correlation Between HPSE-1 Expression and Incubation Time Using a Brain Slice Model

Immunohistochemistry was performed using OCT-embedded sections of brain slices, which had melanoma cells (brain-metastatic B16B15b-EGFP) implanted and incubated for 2, 12, and 24 h, respectively. Upon staining for HPSE-1 using an anti-HPSE-1 polyclonal antibody, we observed low levels of HPSE-1 staining on B16B15b-EGFP cells that had been implanted on brain slices for 2 h. However, when cells were incubated for 12 h, they showed a distinct increase in HPSE-1 staining and an augmented invasive capability. Finally, a 24-h time determination showed heaviest HPSE-1 staining as well as greatest amount of cell invasion (Fig. 1).

HPSE-1 Increases the Number of Invading Cells Using a Brain Slice Model

Endogenous HPSE-1 levels in cells are relatively low and only after 24-h of incubation, HPSE-1 activity can be significantly detected, thus influencing cell invasion [Marchetti et al., 1993, 1996; Nakajima et al., 2001]. To determine the effect of HPSE-1 on brain-metastatic melanoma invasion, B16B15b-EGFP cells were pre-treated with HPSE-1 at several concentrations (0–50 ng/ml), implanted onto orthotopic brain slices, and incubated for 72 h. Immunohistochemistry was subsequently performed to visualize GFP using an anti-GFP polyclonal antibody on OCT-embedded brain slice sections. Since B16B15b-EGFP melanoma cells were stably transfected with EGFP, EGFP staining was a decisive way to mark invading cells (Fig. 2). Controls consisted of using (1) the non brain-metastatic cell line B16F10 [Fidler, 1973; Marchetti et al., 1993], (2) an alternative heparan sulfate (HS) degradative enzyme, heparitinase-III (HepIII), which cleaves HS differently than HPSE-1 [Ernst et al., 1995; Vlodavsky and Friedmann, 2001], and (3) a specific HPSE-1 inhibitor, suramin, at a concentration (100 μM) known to completely inhibit HPSE-1 [Marchetti et al., 2003; Roy et al., 2005]. These control experiments were inhibitory of invasion (Fig. 4A,B).

By analysis of serially cut brain sections, B16B15b cells pre-treated with HPSE-1 showed a twofold increase in the number of invading cells compared to corresponding control groups (Fig. 3). Statistical analysis of both HPSE-1

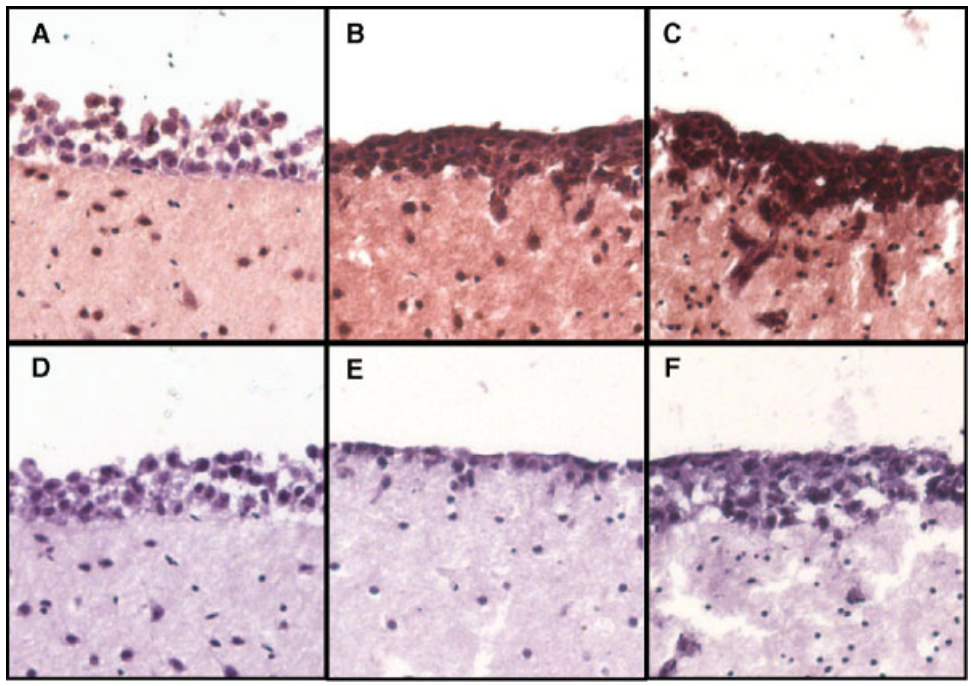

Fig. 1. Heparanase (HPSE-1) time-dependent expression in brain-metastatic melanoma cells. B16B15b-EGFP cells were implanted on brain slices, and following a 2 h (**A**, **D**), 12 h (**B**, **E**), and 24 h (**C**, **F**) post-implantation, experiments were terminated, and immunohistochemistry (IHC) was performed. A–C: IHC staining with anti-HPSE-1 polyclonal antibody showing an augmented HPSE-1 expression at increasing time points. D–F: Negative control (no HPSE-1 antibody present). Digital images were produced using an Axioplan microscope with Advanced Spot imaging program (Diagnostic Instruments, Sterling Heights, MI) with 20x objective using identical conditions for all photographs.

treated B16B15b and control cell populations indicated a correlation between the number of cells invaded and cell invasion depth (Table I). This correlation was further analyzed and determined to be a good predictive model for overall cell invasion. Analysis of covariance showed a significant difference of invasiveness in HPSE-1 treated B16B15b cells when compared to corresponding control groups (Table I). Furthermore, we could not detect any difference in HPSE-1-mediated invasion following plating B16B15b-EGFP cells in different domains of brain affected by brain-metastatic processes (data not shown).

DISCUSSION

We provide first time evidence demonstrating that heparanase (HPSE-1) increases in vivo invasiveness of brain-metastatic melanoma cells using a brain slice model.

HPSE-1 is a unique mammalian HS degradative enzyme involved in degrading and remodeling ECM and basement membranes (BM), and a molecular determinant of cancer metastasis in a variety of tumor types [Nakajima et al., 1988; Parish et al., 2001; Vlodavsky and Friedmann, 2001]. Such invasive capabilities of HPSE-1-expressing tumor cells can be due to the enzyme's involvement in ECM degradation and remodeling. HS binds to various constituents of the ECM and hence plays important roles in maintaining ECM integrity [Bernfield et al., 1999; Iozzo, 2005]. Heparanase, which degrades HS chains of HSPG, compromises this structural integrity. The result is that HSPG are degraded so that invasion and metastatic spread occurs [Goldshmidt et al., 2002; Reiland et al., 2004]. Purified recombinant HPSE-1 in cell suspension degraded the BM and ECM of brain slices compromising the integrity of the brain tissue. Our results further confirm in an in vivo setting the relevance of HPSE-1 as an important modulator of tumor cell invasion into the brain.

Mechanisms by which HPSE-1 facilitates cancer progression likely involves more than just degrading and compromising ECM structural integrity, including complex interactions between tumor cells and the host tissue environment. This was further confirmed by performing

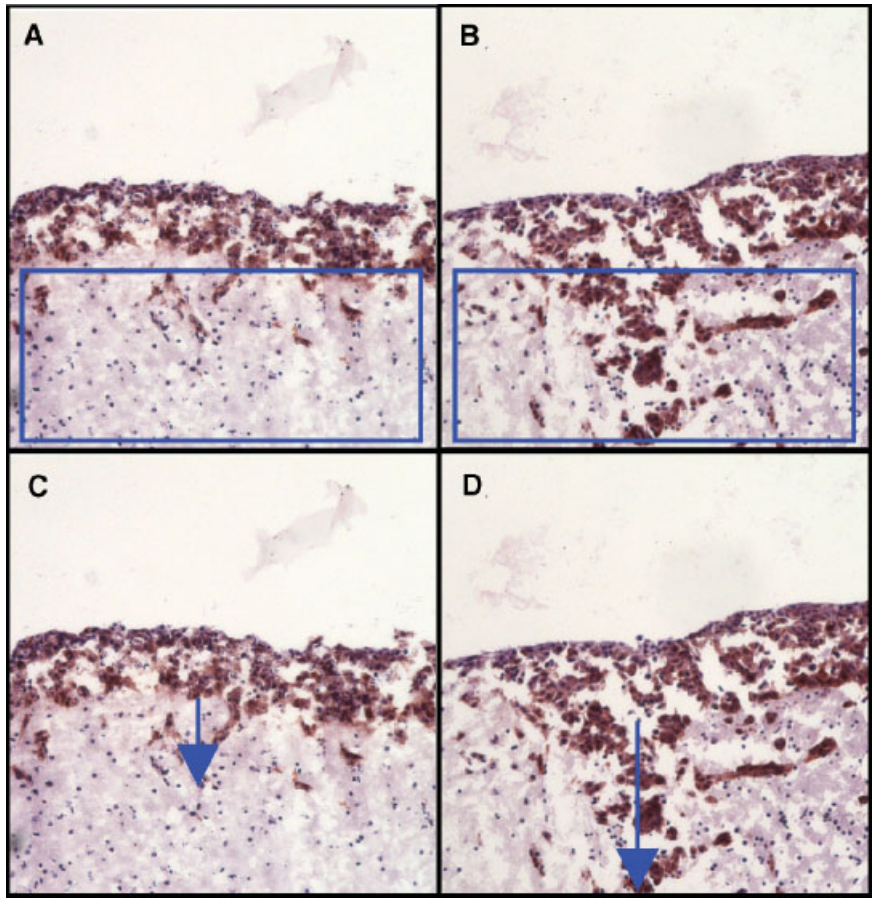

Fig. 2. Representative experiment showing tumor cell invasion by using a brain slice model. Murine brain-metastatic melanoma cells, stably transfected with enhanced green fluorescent protein (B16B15b-EGFP), were plated on the caudate putamen of an organotypic brain slice. After 72 h of incubating the cells on brain slices, they were formalin-fixed, frozen, sectioned further, and IHC stained using an anti-GFP antibody as a melanoma cell marker. (**A**, **C**) Cells plated with no treatment. (**B**, **D**) cells plated after treatment with heparanase (50 ng/ml). (A, B) Area (12 μm^2) used to count the number of invading cells. (C, D) Arrows in plates illustrate how invasion depth was measured.

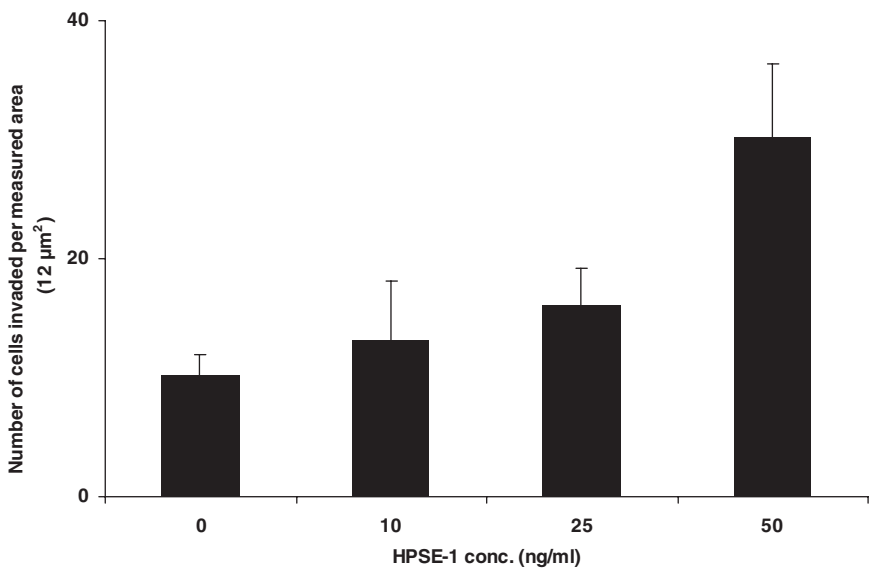

Fig. 3. Extent of invasion into brain slices by murine brain-metastatic melanoma cells (B16B15b) using quantitative analysis at 72 h post-implantation. Invasion by EGFP-expressing cells was measured by the number of cells invading beyond the surface of the brain per unit of area. B16B15b cells pre-treated with heparanase (HPSE-1) at increasing concentrations (0–50 ng/ml) showed a correlative greater number of cells invading into the brain slice. See ''Materials and Methods'' section for experimental details.

TABLE I. Statistical Analysis of Invasion Quantitation Using a Brain Slice Model

Cell treatment	Pearson correlation[a]	Regression analysis[b]	ANCOVA[c]	Tukey grouping[d]
Control	0.59430	0.3532	0.99901	A
Heparanase	0.59155	0.3499	0.86504	B

[a]Pearson correlation was used to determine the correlation between the number of invading cells and cell invasion depth.
[b]Regression analysis, showing R-square values, was used to determine if this method of analysis makes a predictive model for tumor cell invasion.
[c]Analysis of covariance was performed looking at heterogeneity of slopes with cells as the covariate.
[d]Tukey's grouping, designated by A and B, shows a significant difference between the treatment groups. $P < 0.05$.

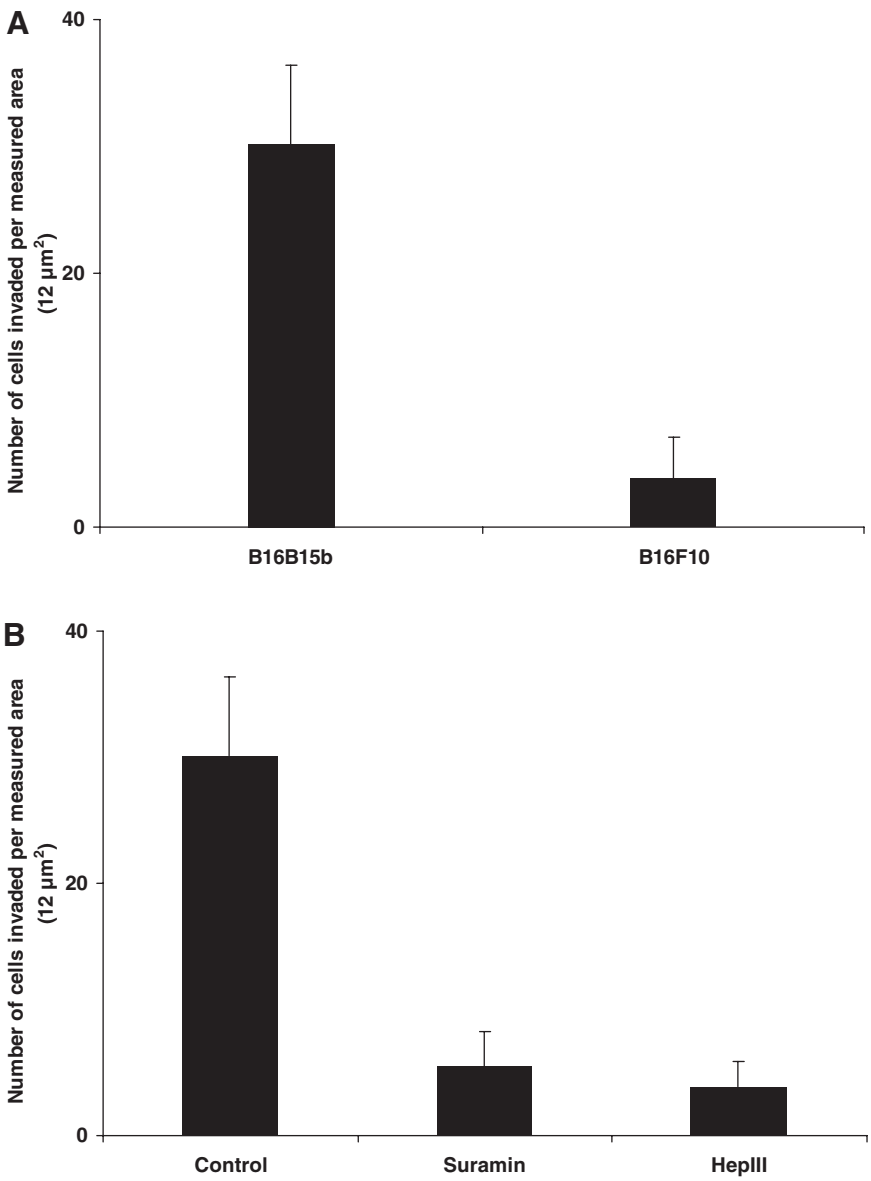

Fig. 4. **A**: Invasion by EGFP-labeled brain-metastatic B16B15b and non-brain invasive B16F10 cells pre-treated with 50 ng/ml heparanase (HPSE-1). **B**: Invasion by EGFP-labeled brain-metastatic B16B15b cells pre-treated with 50 ng/ml HPSE-1 (control), treated with HPSE-1 inhibitor suramin (100 µM), or pre-treated with 50 ng/ml heparitinase-III (HepIII). See ''Materials and Methods'' section for experimental details.

time-course experiments where HPSE-1 expression was augmented with increased time implantations of untreated brain-metastatic melanoma cells (Fig. 1). During our time-course experiments, we also observed HPSE-1 staining in cells within the area of normal brain tissue. Our laboratory has previously demonstrated HPSE-1 production by astrocytes, which contribute to the brain-metastatic specificity of melanoma cells [Marchetti et al., 2000].

Importantly, the orthotopic brain slice model presents an in vivo model of the central nervous system in which neuronal architecture is maintained. It proves to be useful in studying both physiological and pathophysiological developmental processes. For example, it has been previously used in studying mechanisms of invasion in glioma and astrocytoma cells [Matzner et al., 1992; Ohnishi et al., 1998; Jung et al., 2001; Yoshida et al., 2002]. In our case, this model can create co-culture settings between melanoma cells and the brain tissue environment that allows for more realistic growth settings yet being relatively easy to manipulate for treatment. First, it provides the ability to analyze processes at the cell and molecular level while the cells are growing in a viable environment. Second, it allows the opportunity to perform invasion assays without having to add chemoattractants to these assays [Stoppini et al., 1991]. Third, it is especially helpful because it is thought that metastasis is dependent on certain host tissue properties, particularly the host microenvironment and its growth factors. For these reasons, this model can be of considerable use in future research efforts to investigate mechanisms underlying brain metastasis in general, brain-metastatic melanoma in particular.

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