Neurotrophin Stimulation of Human Melanoma Cell Invasion: Selected Enhancement of Heparanase Activity and Heparanase Degradation of Specific Heparan Sulfate Subpopulations

Dario Marchetti, David J. McQuillan, William C. Spohn, Dan D. Carson, and Garth L. Nicolson

Departments of Tumor Biology [D. M., W. C. S., G. L. N.] and Biochemistry and Molecular Biology [D. D. C.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, and Institute of Biosciences and Technology, Texas A & M University, Houston, Texas 77030 [D. J. M.]

ABSTRACT

Heparanase is an endo-β-D-glucuronidase, the enzymatic targets of which are the glycosaminoglycan chains of heparan sulfate proteoglycans. Elevated levels of heparanase are associated with the metastatic potential of melanoma cells. Treatment of murine and human melanoma cells with the prototypic neurotrophin nerve growth factor (NGF) increases the production of heparanase by melanoma cells. We reported previously that physiological concentrations of NGF increased in vitro Matrigel invasion of early-passage human brain-metastatic 70W melanoma cells but not melanoma cells metastatic to other sites or nonmetastatic melanoma cells. Here we found that treatment of 70W melanoma cells with neurotrophin NT-3 increased Matrigel invasion, whereas treatment with neurotrophins other than NGF or NT-3 did not influence invasion. Mutants of NGF that do not bind to the neurotrophin receptor p75NTR or other nonneuronal growth factors were not able to enhance the invasion of 70W melanoma cells. When 70W cells were exposed to antisense oligonucleotides directed against p75NTR mRNA, there was a reduction in NGF and NT-3 binding, and the neurotrophins failed to enhance Matrigel invasion. To study the properties of heparanase in NT-regulated malignant melanoma invasive processes, we developed a sensitive heparanase assay consisting of purified [35S]heparan sulfate subpopulations separated by agarose gel electrophoresis. Incubation of 70W cells with NGF or NT-3, but not brain-derived NT factor, NT-4/5, or mutant NGF, resulted in increased release of heparanase activity that was capable of degrading a subpopulation of heparan sulfate molecules.

INTRODUCTION

The vascular endothelium and its underlying BM make up an important barrier that tumor cells must penetrate to colonize distant organs (1). The major components of BM include type IV collagen, laminin, entactin, and HS proteoglycans (2). During tumor invasion, the BM becomes disorganized due to changes in the expression of BM constituents, alterations in their assembly, and enzymatic degradation (1, 3–5).

Degradation of BM components by invading malignant cells can be affected by paracrine or trophic factors in the organ microenvironment (6). For example, brain-matrinic murine and human melanoma cells respond to certain NTs by increasing their production of ECM-degrading enzymes (5, 7, 8). NTs, including NGF, NT-3, BDNF, and DDH2, O, double-distilled water; HSPG, heparan sulfate proteoglycan; CNS, central nervous system; HPLC, high-pressure liquid chromatography.

... and also to specific high-affinity NTR represented by the tyrosine kinase family of TRK receptors (9, 11). There is some specificity of NT binding to TRK receptors; for example, NGF shows preferential binding to TrkA receptors, BDNF to TrkB receptors, and NT-3 to TrkC receptors (12).

Malignant melanoma cells express p75NTR in relation to their malignancy and ability to metastasize to the brain (7, 8) within regions that synthesize and respond to NTs (13). We found that overexpression of p75NTR in brain-metastatic melanoma cells correlates with an increase in Matrigel invasion and secretion of ECM-degrading enzymes (7, 8). Although the human brain-metastatic melanoma cells that we examined did not express trkA-encoded p140PS A, they did express trkC-encoded p145VAC, the putative NTR for NT-3 (8). These observations have been confirmed using clinical specimens from patients with metastatic melanomas at different stages of tumor progression by in situ hybridization and immunohistochemistry. An inverse relationship was found between expression of NT and NTR at the invasion front of human melanoma brain metastases (14).

Important BM degradation targets of invading melanoma cells are the HS chains found on HS proteoglycans (5, 7). HS is produced by a wide variety of different cell types and is found at the external surfaces of cells and in BMs and other ECMs (15, 16). The major HS proteoglycan of the BM, perlecan (17), is abnormally expressed by melanoma cells (18, 19). Malignant cells can also modulate the expression of HS proteoglycans by releasing degradative enzymes, such as heparanase (3), or cytokines can influence the turnover and biosynthesis of host cell HS proteoglycans (20).

Here we report that invasion of brain-metastatic melanoma cells through Matrigel is selectively augmented when these cells are exposed to NGF or NT-3 but not BDNF, NT-4/5, or mutated NGF and other nonneuronal factors. Using antisense oligonucleotides derived from the p75NTR sequence, we demonstrated the biological relevance of p75NTR in the invasion process. Additionally, by development of a sensitive heparanase assay that separates purified [35S]HS species by agarose gel electrophoresis, we report that expression of heparanase is up-regulated by NGF and NT-3 and that heparanase preferentially degrades specific HS species.

MATERIALS AND METHODS

Chemicals. Heparin, HS, chondroitin sulfate C, and t-saccaride 1,4-lactone were acquired from Sigma Chemical Co. (St. Louis, MO). [3H]Heparin (0.44 mCi/mg) and [35S]Sulfate (43 Ci/mg) were purchased from DuPont-New England Nuclear (Wilmington, DE) and ICN Biochemical (Irvine, CA), respectively. FBS and DMEM were purchased from Gibco. Inc. (Grand Island, NY), guanidine hydrochloride from Life Technologies, Inc. (Gaithersburg, MD), and 3-[3-(chomiamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid from Boehringer Mannheim (Indianapolis, IN). Suramin was a generous gift from Dr. Motowo Nakajima (Ciba-Geigy Japan Limited, Takarazuka, Japan).

Tissue Culture. Early-passage human A-875, MeWo, and its wheat germ agglutinin-resistant 70W melanoma cell lines (21) were maintained as monolayer cultures in 1:1 (v/v) DMEM:F12 medium (Life Technologies, Inc.) supplemented with 5% FBS (Gibco, Inc.) at 37°C in a humidified 5% CO2-
95% air atmosphere. The cells were subcultured every 3–4 days by trypsin-EDTA treatment, except for the 70W cell line, the medium of which was changed every 24 h. Murine melanoma B16 cells (parental F1, metastatic-BL6, and brain-metastatic-B15b; Refs. 3 and 36) of less than eight passages from an original frozen stock were grown to subconfluence in a 1:1 (v/v) mixture of DMEM:F12 medium supplemented with 5% FBS. Cells were harvested by rinsing them twice in medium without serum, followed by treatment for 10 min with 2 mM EDTA in Ca2+/-Mg2+-free Dulbecco’s PBS. All cell lines were subcultured when they reached 60–80% confluence. One day before NT (0–4 h) treatment, the medium was replaced with serum-free medium (7). The human RL95 cell line, an adenocarcinoma of the uterus (22), was chosen as a source of HS proteoglycan because more than 95% of the GAGs made by these cells is HS (23). These cells were grown in 1:1 (v/v) DME:F12 supplemented with 10% FBS (v/v). The [35S]GAG labeling required the use of special medium that was sulfate deplete (RPMI 1640, formula 79–5139 EC; Life Technologies, Inc.). All cell lines were periodically checked for Mycoplasma contamination using a Geneprobe kit (San Diego, CA), and only Mycoplasma-free cells were used.

Chemoimmun and Heparanase Assays. Tumor cell invasion following NT treatment was assayed by use of Transwell (Costar, Cambridge, MA) cell culture chambers with Matrigel-coated filters, and invasion was measured by microscopy using a Cytofluor Model 2300 (Millipore, Bedford, MA; Ref. 7), taking into consideration both background and map reading area of the instrument. Heparanase activity was determined by degradation of [35S]HS using high-speed gel permeation chromatography (24) with some modifications (7). Briefly, subconfluent cells, whether human (70W) or murine (B16BL6 and B16B15b), were harvested and solubilized in 50 mM Tris-HCl (pH 7.5), 0.05% NaNO3, and 0.5% Triton X-100 for 30 min at 4°C. Cell lysates were then centrifuged at 12,000 × g for 30 min at 4°C and concentrated by means of Amicon-30 microconcentration units (Amicon, Beverly, MA), according to the manufacturer’s instructions. Cell lysates (50–70 µg protein) were then incubated with radiolabeled HS and 0.2 mM sodium acetate (pH 5.0) for 18 h at 37°C (final reaction volume, 10–100 µL). Reaction was terminated by heating samples for 15 min at 95°C. Additionally, a delipidation step was applied to cell lysates following the heparanase assay and before HPLC analysis. A TSK gel G3000 PWX2 column (7.8 mm × 30 cm; 6-µm particle size) from Tosohaas, Inc. (Montgomeryville, PA) was used in the high-speed gel permeation chromatography.

NT Indination and Receptor Binding Assays. Purified recombinant human NGF, BDNF, and NT-3 were iodinated by using an accepted procedure (25) using Na[125I]-carrier-free (Amersham, Arlington Heights, IL) and treatment with matrix-bound lactoperoxidase/glucose oxidase using the Enzymobead reagent (Bio-Rad, Richmond, CA). Indination was previously shown not to interfere with NT bioactivity (25). Briefly, beads were rehydrated overnight at 4°C, centrifuged, and resuspended in 190 µL of ddH2O. The Enzymobead suspension (50 µL) was added to the indination reaction, as described by the manufacturer, at pH 6.0. An additional 50 µL of the resuspended beads in ddH2O were added, and incubation occurred for another 2 h. The mixture was incubated for 2 h at room temperature (25°C), and the labeled NTs were separated from free iodine using disposable desalting columns (Pierce, Rockford, IL). Usually, NTs (2 µg) were incubated in the presence of 1.5 mCi Na[125I], and preparations with specific activities of 3500 cpm/nmol were typically obtained. The [125I]NTs were stored at 4°C and used within 2 weeks of preparation. NTs were extracted by incubating the cells with continuous gentle mixing in 2% NP40 in PBS for 1.5 h at 4°C. The samples were centrifuged to remove insoluble material, and each pellet was resuspended in NP40 in PBS and centrifuged a second time. The supernatants were combined and diluted 1:1 with 20 µg/ml cytochrome C in PBS containing 0.5% (v/v) NP40. Receptor binding assays were subsequently performed with appropriate controls as described elsewhere (7, 26).

Cell Treatment with Antisense Oligonucleotides. Nucleic-resistant phosphorothioate 21-mer oligonucleotides were synthesized corresponding to the 5’-translation initiation site of p75NT (27). Thioate linkages were placed at each end of the primers with internal thioates to prevent rapid degradation and binding to noncomplementary sequences. The oligonucleotide sequences were as follows: p75NT antisense, 5’-GGGCGACCTGGCCCTCCATGCCG-3’; p75NT sense, 5’-GGGCGATGGGGCAGGTTGCA-3’ (Genosys Biotechnologies, The Woodlands, TX). Oligonucleotides were added to a final concentration of 5 µM directly to the cells in 24-well plates (1.2 × 105 cells/well) every 24 h for 4 days in serum-free medium. Cells were then transferred onto invasion chambers (2 × 105 cells/filter), and invasion assays were performed as described previously (7) in the presence of 2 nM human NGF or NT-3 and the corresponding oligonucleotide. NTR were measured as described above.

HS and Identification of Cell Surface HS Components. RPMI 1640 was used as the basal medium for [35S]Sulfate radiolabeling of human RL95 adenocarcinoma cell HS subpopulations (22). Briefly, near-confluent RL95 cells were rinsed several times with serum-free RPMI 1640 (minus sulfate) supplemented with 3.3 mM MgCl2, 1.2 g/l NaHCO3, 15 mM HEPES (pH 7.2), 2.5 units/ml penicillin, and 2.5 µg/ml streptomycin sulfate. Streptomycin sulfate served as the sole source of nonradioactive sulfate in this medium (final concentration, ~2 µM). The cells were incubated overnight in the same low-sulfate medium described above containing 0.5 µCi/ml [35S]Sulfate. The medium was collected, and the cell monolayers were rinsed several times with ice-cold PBS. The cell monolayers were then incubated for 30 min on ice with PBS containing 50 µg/ml trypsin to release cell surface proteoglycans. Cells did not detach from the tissue culture surfaces under these conditions, nor was cell viability compromised as assayed by trypan blue dye exclusion. The material released into the “trypsinate” was collected and placed in a boiling water bath for 5 min and centrifuged on ice to inactivate the trypsin.

The released material was extensively dialyzed for 96 h against ddH2O, and aliquots were examined before, during, and after each dialyzing step until no free sulfate was present. Trypsin-resistant [35S]HS from RL95 cells was prepared similarly with some modifications that consisted of scraping cells off the plates in the presence of PBS and adding an equal volume of 20% trichloroacetic acid (w/v), 6% phosphotungstic acid (w/v) and placing them on ice for 30 min, followed by centrifugation at 2000 × g for 10 min. Precipitates were first centrifuged at 10,000 × g for 10 min, and the pellets were resuspended and centrifuged two more times in 5 ml 10% trichloroacetic acid.

The final pellet was resuspended in 2 ml 0.1 M Tris-HCl, 5% (v/v) ethanol, and 2 mM CaCl2 (pH 8.0). After boiling for 2 min and cooling to room temperature, the samples were either incubated overnight with pronase (10 mg/ml; Refs. 48 and 49) or they underwent alkaline borohydride treatment (8-elimination) at 45°C in the presence of 0.05 M NaOH and 1 m sodium borohydride for 24 h, followed by neutralization with acetic acid (22). Precipitates were collected, and supernatants were dialyzed extensively with ddH2O. Aliquots were removed, radioactivity was determined, and specific activity was calculated.

Analytical Column Chromatography and HS Chemical Analyses. A Superose 6 column (1.0 × 30 cm; Pharmacia LKB, Upsala, Sweden) was eluted with 4 mM guanidine hydrochloride, 0.5% (w/v) [3-(3-chomaidopropylidemethylammonio)]-2-hydroxy-1-propanesulfonic acid, and 50 mM sodium acetate (pH 6.0) at a flow rate of 0.4 ml/min, and 0.4-ml fractions were collected. Aliquots were taken for determination of radioactivity by scintillation counting. Molecular size estimates for GAG chains were based on the method of Wasteson (30) for Sepharose 6FB. The accuracy of molecular weight determination by Superose 6 gel filtration for CS chains was assessed by direct comparison with a Sepharose 6B column calibrated with CS chains of known molecular mass (31). For HS chains (derived from alkaline-borohydride cleavage) and HS glyccopeptides (derived from pronase digestion of trypsin-released HS proteoglycan), the calibration held for similarly sulfated GAGs. Analyses involving standard nitric acid hydrolysis (32) were performed to confirm labeling of heparan sulfate chains on GAGs. Briefly, samples for digestion were added to 0.5-ml beakers containing 1% (w/v) n-butyl nitrite (in 95% ethanol). The mixture was incubated at 37°C for 15 min at room temperature (25°C) to dissociate [35S]-labeled digestion.
products from molecular weight complexes (data not shown). Following this step, agarose gel (1.2% w/v) electrophoresis was performed at 75 V for 1 h at 25°C or until the samples migrated approximately two-thirds of the entire gel length. Autoradiography was performed on the dried gel by exposure to X-AR 5 film (Kodak, Rochester, NY) for 3–7 days. The direction of electrophoretic mobility shown in the figures is from top to bottom in all cases.

In the sequential agarose gel-Superose 6 chromatographic analysis, gel pieces from the slower-migrating band of [35S]HS and the intact HS band following agarose gel electrophoresis were soaked in 2 ml of Tris-borate-EDTA (TBE) buffer for 2 h, placed in small dialysis bags, and electroeluted for 4 h at 50 V using a Minigel apparatus. Liquid was collected from the bag, spun in an Eppendorf centrifuge, and concentrated. Radioactive material then was loaded onto a Superose 6 column and eluted under dissociating conditions; then fractions were collected.

RESULTS

Effects of NTs on In Vitro Invasion of Melanoma Cells. Using Matrigel-coated filters in Transwell invasion units, the effects of several NTs on the invasive capacity of the brain-metastatic melanoma cells were determined. Previously, we found that highly metastatic human 70W melanoma cells were stimulated to invade the Matrigel barrier by NGF at higher rates than the other lines tested (7, 8). This increase in invasion has been shown to be associated with high levels of p75NT receptors, cell surface NTR molecules, and NTR sites/cell (7, 8). We have expanded these results to include other NTs. Melanoma cells were incubated with HPLC-purified preparations of human NT at optimal concentrations to saturate NTRs (NGF, BDNF, NT-3, or NT-4/5). Only NGF and NT-3 stimulated invasion of 70W cells (Fig. 1). In contrast, none of the NTs had dramatic effects on the MeWo parental line. Additionally, NT stimulation of ECM invasion was specific, since other factors such as keratinocyte growth factor and Kaposi’s sarcoma-derived fibroblast growth factor, which are not present in the brain microenvironment, did not stimulate invasion (data not shown). The results were consistent with the presence of the appropriate NTR (p75NT and TrkC but not TrkA) in these cells (8). When we used an NGF mutant in which alanine residues replaced lysine-32, lysine-34, and glycine-35 (33), we found significantly lower degrees of invasion than with wild-type NGF (Fig. 1). This NGF mutant has less than 1% of the binding capacity of NGF to p75NT but retains 65% of the specific biological activity of NGF and, importantly, binds to the TrkA receptor with only a 2-fold reduction in affinity (33). The result indicates that binding of NTs to the low-affinity p75NT receptor is necessary for stimulation of 70W cell invasion. Incubation of the 70W cells with higher concentrations of NTs (100 ng/ml) resulted in reduced rates of invasion, as observed previously with NGF (7, 8).

We further investigated the role of p75NT in the invasion of 70W cells by using specific antisense oligonucleotides designed using the S'-translated region of human p75NT (27). Analysis of cell growth indicated that addition of these oligonucleotides to the medium of 70W cells did not affect their rate of proliferation (data not shown). In contrast, incubation of NT-treated 70W cells with p75NT-specific antisense oligonucleotides prior to (96 h) and during (72 h) chemoinvasion assays resulted in inhibition of invasion in the presence of NGF or NT-3 (Fig. 2). In contrast, a p75NT sense construct had little effect on the rates of invasion stimulated by NGF or NT-3 (Fig. 2).

Inhibition of NT Binding to p75NT by Antisense Oligonucleotides. Previously, we found that p75NT is overexpressed in 70W cells (8), resulting in increased NGF binding to these cells (7). We, therefore, examined NT-3 binding to 70W cells and whether the inhibition of invasion observed by the addition of antisense oligonucleotides correlated with reduction of NT-3 receptors. Table 1 shows the results of binding assays of human [125]NTs to membrane preparations from 70W, MeWo, and A-875 cells. A-875 is a brain metastasis-derived human melanoma cell line known to express high levels of p75NT (0.5 × 10⁶ receptors/cell) but not TRK receptors (34, 35). Binding of [125]NT-3 to 70W, MeWo, and A-875 cells was observed and compared with binding of [125]NGF and [125]BDNF, respectively. The brain-metastatic A-875 and 70W cells bound higher
Table 2 Comparison of antisense or sense p75<sup>NTR</sup> treatment on binding of human [125<sup>I</sup>]NGF or [125<sup>I</sup>]NT-3 to human 70W melanoma cells

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<th>Time (days)</th>
<th>Antisense p75&lt;sup&gt;NTR&lt;/sup&gt; (fmoles [125&lt;sup&gt;I&lt;/sup&gt;]NT-3 bound/mg protein)</th>
<th>Sense p75&lt;sup&gt;NTR&lt;/sup&gt; (fmoles [125&lt;sup&gt;I&lt;/sup&gt;]NT-3 bound/mg protein)</th>
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- ND, not determined.

Fig. 3. Superose 6 chromatographic analysis of [35S]HS subpopulations before and after nitrous acid degradation. HS radiolabeling, isolation (cell-associated or secreted) from RL95 cells, and subsequent chromatographic analyses were performed as described in "Materials and Methods." Column calibration was performed with CS chains of defined molecular weight. Peak elution position (K<sub>d</sub>) was calculated relative to void (V<sub>v</sub>, 7.6 ml) and total (V<sub>t</sub>, 22 ml) column volumes by the formula K<sub>d</sub> = (V<sub>c</sub> - V<sub>v</sub>)/(V<sub>c</sub> - V<sub>t</sub>). Cell-associated [35S]HS from RL95 eluted in two peaks (Peak I, K<sub>d</sub> = -0.27; Peak II, K<sub>d</sub> = -0.77); nitrous acid degradation of cell-associated [35S]HS secreted by RL95 into medium; O, [1<sup>H</sup>]heparin (M<sub>r</sub> ~70,000). Additional [35S]-labeled material was retarded in the column and eluted at a median K<sub>d</sub> of 0.77 (Fig. 3, Peak II). We next compared some of the properties of the high molecular weight HS material with those of the low molecular weight HS. Preparative Superose 6 chromatography was performed, and the two HS subpopulations were separated and pooled: peak I (fractions 25–34) represent-

made use of RL95 cells as a HS source, the rationale being that RL95 are able to grow in sulfate-depleted media and most (>95%) of GAG chains synthesized by these cells is HS, thus providing a convenient substrate to investigate heparanase action and heparanase regulation by NTs. RL95 were incubated with [35S]sulfate, and selected HS subpopulations were obtained as described in "Materials and Methods." Cell lysates were dialyzed to remove unincorporated [35S]. The original [35S]HSPG populations underwent extensive pronase digestion and alkaline borohydride treatment (β-elimination) to digest the polypeptide backbone while leaving the GAG chain portion intact (38, 39). Nitrous acid digestion, followed by Superose 6 chromatography of metabolically labeled GAG populations (3–9 × 10<sup>6</sup> dpm/μg HS), confirmed the identity of these GAGs as HS (Fig. 3). The material was also sensitive to bacterial heparitinases (23) but resistant to chondroitinases AC, C, and ABC digestion (data not shown). Superose 6 chromatography then was performed on the same material using columns calibrated with reference fractions of radiolabeled CS (30). Most of the [35S]HS molecules were of high molecular weight and eluted as a relatively sharp peak with a median K<sub>d</sub> of 0.27 (Fig. 3, Peak I; M<sub>r</sub> ~70,000). Additional [35S]-labeled material was retarded in the column and eluted at a median K<sub>d</sub> of 0.77 (Fig. 3, Peak II). We next compared some of the properties of the high molecular weight HS material with those of the low molecular weight HS. Preparative Superose 6 chromatography was performed, and the two HS subpopulations were separated and pooled: peak I (fractions 25–34) represent-

Fig. 4. Agarose gel electrophoresis of cell-associated [35S]HS indicating high (M<sub>r</sub> ~70,000: 70 kDa) and low (M<sub>r</sub> ~23,000: 23 kDa) fractions of [35S]HS. Estimation of molecular sizes is based on elution profiles from Superose 6 column calibrated with standard glycans, as mentioned in Fig. 3.
Heparanase Regulation in Human Malignant Melanoma

The Peak I HS Component Is Degraded by Heparanase. Since peak I, the high molecular weight [35S]HS component, was apparently degraded by heparanase, we performed several controls to insure that degradation was indeed due to heparanase enzymatic action. For example, we considered that different molecular weight HS might arise artificially after binding cellular components in the cell extracts due to the effects of the electrophoresis (40, 41). To test this, we concentrated cell extracts from brain-metastatic human melanoma cells (VOW) and mixed them with the high molecular weight [35S]HS, and the mixture was then immediately electrophoresed. In addition, high molecular weight [35S]HS was incubated at 37°C for 18 h under the conditions used for the heparanase assay (7) but in the absence of heparanase, and the material migrated similarly to intact HS (Fig. 5A, Lanes 1 and 2). Additional controls consisted of mixing [35S]HS with heat-inactivated cell lysates. Excess nonradioactive CS type C (300 µg/ml) was added to the cell lysates and [35S]HS to minimize any effects due to nonspecific interactions of the GAG chains with components of the cell extracts (40). None of these procedures changed the migration pattern of the HS (data not shown). Experiments using extracts of murine melanoma cell lines known to express different amounts of heparanase were used (24, 36). The high molecular weight [35S]HS with various cell lysates resulted in degradation of HS, especially when murine brain-metastatic melanoma (B16-B15b) cell lysates were used; RL95 [35S]HS was incubated with B16B15b cell extracts in the absence or presence of 20 mM D-saccharic acid 1,4-lactone, a potent exo-ß-glucuronidase inhibitor (42, 43), with GAG fragments analyzed by both high-speed gel permeation chromatography and electrophoretic analysis. We did not observe a significant inhibition of GAG degradation. Conversely, incubation of the same cellular extracts with heparin or suramin (100 µM), potent inhibitors of melanoma heparanase and invasion (44), abrogated GAG degradation, confirming a heparanase action (data not shown). We, therefore, examined whether heparanase activity in 70W cells could be NT modulated, similar to the results seen for NGF (7). Human melanoma cells (70W) were incubated with each NT member and NGF triple mutant (p75NTR binding-deficient); then cell lysates were prepared, incubated with high molecular weight [35S]HS, and electrophoresed. In comparison to known NGF-driven heparanase up-regulation, a marked decrease of intact

Fig. 5. A, agarose gel electrophoresis of purified high molecular weight [35S]HS (peak I). Gel electrophoresis was performed as described in “Materials and Methods.” Electrophoresis shown is from top to bottom. Lane 1, cell surface [35S]HS incubated with 70W cell lysate and immediately electrophoresed (time 0). Lane 2, cell surface [35S]HS incubated with 70W cell lysate for 18 h following heparanase inactivation by heating sample at 95°C for 15 min. B, cell surface high molecular weight [35S]HS analysis following incubation with extracts from human brain-metastatic cells (70W) not exposed (Lane 1) or previously exposed to NGF (Lane 2) or NT-3 (Lane 3). C, agarose gel electrophoresis of heparanase-exposed high molecular weight [35S]HS (Lane 1) following exposure of human brain-metastatic melanoma cells (70W) to BDNF (Lane 2), NT-4/5 (Lane 3), or NGF triple mutant (Lane 4).
[35S]HS (Fig. 5B, Lane 1) was observed with a concomitant appearance of low molecular weight 35S-labeled material, if the cells were exposed to NGF or NT-3 (Fig. 5B, Lanes 2 and 3). Treatment of 70W cells with other NTs, i.e., BDNF or NT-4/5, did not result in increases of heparanase activity, as seen by degradation of the intact high molecular weight [35S]HS material (Fig. 5C). Elution of the heparanase-digested and aggregated material from the agarose gel, followed by Superose 6 chromatography under dissociating conditions (4 M guanidine-HCl), resulted in a lower molecular weight peak compared to the starting material (Fig. 6). That this was indeed the result of heparanase action and not the result of other enzymatic activities, such as sulfatases, was sustained by the fact that we did not observe any detectable radioactivity at the V, region of the column (Fig. 6). Additional confirmation came following BioGel P-2 column chromatography of our cell lysate after reaction with [35S]HS, using in parallel CS di-, tetra-, and octasaccharides as well as labeled free sulfate as molecular weight standards. There was no appreciable radioactivity detected in the region corresponding to the migration position of free sulfate (data not shown).

We next compared heparanase cleavage of our high molecular weight-purified [35S]HS with bovine lung [3H]HS (24, 37); both were sensitive to heparanase as well as bacterial heparitinases (22, 24, 37). We obtained indications regarding their charge densities by means of an anion-exchange column (DEAE-Sepharose) chromatography after equal loading of radiolabeled material and their separation (38). Their elution profiles were distinct in relation to both elution positions and peak heterogeneity, as evidenced by a different sulfation pattern between the two substrates (RL95 HS was less sulfated than bovine lung HS; data not shown). GAGs from these two sources were added to murine metastatic B16BL6 cell lysates because this B16 subline is already known to contain high amounts of heparanase (3). The heparanase-digested [3H]HS was shifted to a higher elution position than the undigested [3H]HS as expected (24, 37); however, heparanase digestion of purified cell surface [35S]HS resulted in a more dramatic shift in the size distribution. Indeed, the use of cell surface [35S]HS strongly enhanced the appearance of intermediate molecular weight fragments (Fig. 7). Similar results were obtained using heparanase from murine brain-metastatic B16-B15b or human brain-metastatic 70W cells (data not shown). Incubation of this cell surface-derived [35S]HS subpopulation with brain-metastatic 70W human melanoma cell extracts resulted in the generation of characteristic lower molecular weight fragments (Fig. 8A). Incubation of 70W cells with human NGF or NT-3, followed by determination of heparanase activity using HPLC analysis, confirmed a selective NT enhancement of heparanase activity in brain-metastatic human melanoma cells, with NT-3 augmenting heparanase activity at levels higher than NGF or untreated cells (Fig. 8A); heparanase degradation potential remained independent of previous exposure of melanoma cells with members of the NT family other than NGF or NT-3, i.e., BDNF, NT-4/5, and the NGF triple mutant molecule (Fig. 8B).

The heparanase degradation potential observed for melanoma cells "in vitro" was confirmed "in vivo" by immunofluorescence studies using clinical specimens from patients with brain-metastatic melanoma by means of purified preparations of monoclonal antibody (10E5 monoclonal antibody) developed against heparanase.

**DISCUSSION**

An important clinical end point in patients with melanoma and other cancers is the formation of metastases in the brain. Tumor cells that gain access to the brain must traverse microvessels at various organ sites, home to and implant in the brain microcirculation, and eventually invade and survive in the brain (6). The microenvironment of the brain is unique, and it probably provides malignant cells that exhibit preferential colonization to different regions of the brain with specific trophic and other organ factors (6, 45). Since melanomas possess the highest frequency of CNS colonization (46), it is likely...
was also characterized by secretion of the ECM degradative enzymes, such as heparanase (7), and $M_\text{r}$ 72,000 type IV collagenase (8). Heparanase preferentially cleaves $\beta$-$\delta$-glucuronosyl-$N$-acetylglucosaminyl linkages of the HS molecule (37). We have found that CNS-metastatic melanoma heparanase is capable of cleaving cell surface-derived $[^{35}\text{S}]$HS subpopulations. This substrate was found to be sensitive to bacterial heparitinases and to possess a different (less sulfated) sulfation pattern when compared to bovine lung HS already used as a heparanase substrate in previous studies (3, 24, 37). Since we previously localized heparanase at the cell surfaces of metastatic melanoma cells (48), this enzyme may degrade the cell surface as well as GAG chains of ECM HS proteoglycans. It is likely that metastatic cells require heparanase as well as other degradative enzymes, such as type IV collagenases, for tumor cell invasion to occur through the vascular endothelial ECM (5). The 70W cells expressed high levels of p75$^{\text{NTR}}$ but did not express trkA (8). We have expanded these studies to include NT-3, BDNF, NT-4/5, and a NGF triple mutant (K32A + K34A + E35A) unable to bind p75$^{\text{NTR}}$ (33). The highly invasive cells expressed elevated levels of p75$^{\text{NTR}}$, and invasion could be inhibited by an antisense oligonucleotide against p75$^{\text{NTR}}$. NGF and NT-3 also stimulated increased heparanase expression and concomitant digestion of cell surface and pericellular HS GAG chains of respective HSPGs. By purification and use of a selected and homogeneous HS population from a cell surface source, we indeed found increased heparanase activity values. Thus, a HS cell surface origin may provide a more suitable and homogeneous substrate to study melanoma heparanase.

The response of brain-metastatic 70W cells to NTs, such as NGF and NT-3, is consistent with their pattern of brain colonization. When injected i.v. into nude mice, brain-metastatic 70W cells colonize the cerebral cortex of the brain (21). This is a major site of NGF and NT-3 secretion and one of the few sites within the brain microenvironment where both NGF and NT-3 are synthesized (49). Preliminary experiments indicate that NGF-treated 70W melanoma cells show increased metastatic capabilities to the cerebral cortex following i.v. injection in nude mice. This suggests that melanoma cells may invade, survive, and grow preferentially in areas within the brain where secretion of NGF and NT-3 occurs. It is possible that the brain uses NT as paracrine survival and growth factors during brain injury and wound healing (50). Support for this notion is that high concentrations of NGF and NT-3 are found in normal brain tissue at the invasion zones of human melanomas in the CNS (14, 51). How NTs induce CNS invasion is unclear, but specific interactions of NT with low-affinity p75$^{\text{NTR}}$ receptor and induction of degradative enzymes appear to be necessary. Studies are presently ongoing to determine exactly the time-dependent release of heparanase into culture media in relation to HS processing, the presence of HS-binding proteins, and NT regulation.

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Neurotrophin Stimulation of Human Melanoma Cell Invasion: Selected Enhancement of Heparanase Activity and Heparanase Degradation of Specific Heparan Sulfate Subpopulations

Dario Marchetti, David J. McQuillan, William C. Spohn, et al.


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