FAST TRACK
Journal of Cellular Biochemistry 106:200–209 (2009)

Journal of Cellular Biochemistry

Cell Surface Heparan Sulfate Released by Heparanase Promotes Melanoma Cell Migration and Angiogenesis

Madhuchhanda Roy[1] and Dario Marchetti[2*]

[1]*Department of Pathology and Laboratory Medicine, University of Wisconsin, Madison, Wisconsin 53706*

[2]*Department of Pathology, Baylor College of Medicine, Houston, Texas 77030*

ABSTRACT

Heparan sulfate (HS) proteoglycans are essential components of the cell-surface and extracellular matrix (ECM) which provide structural integrity and act as storage depots for growth factors and chemokines, through their HS side chains. Heparanase (HPSE) is the only mammalian endoglycosidase known that cleaves HS, thus contributing to matrix degradation and cell invasion. The enzyme acts as an endo-β-D-glucuronidase resulting in HS fragments of discrete molecular weight size. Cell-surface HS is known to inhibit or stimulate tumorigenesis depending upon size and composition. We hypothesized that HPSE contributes to melanoma metastasis by generating bioactive HS from the cell-surface to facilitate biological activities of tumor cells as well as tumor microenvironment. We removed cell-surface HS from melanoma (B16B15b) by HPSE treatment and resulting fragments were isolated. Purified cell-surface HS stimulated in vitro B16B15b cell migration but not proliferation, and importantly, enhanced in vivo angiogenesis. Furthermore, melanoma cell-surface HS did not affect in vitro endothelioma cell (b.End3) migration. Our results provide direct evidence that, in addition to remodeling ECM and releasing growth factors and chemokines, HPSE contributes to aggressive phenotype of melanoma by releasing bioactive cell-surface HS fragments which can stimulate melanoma cell migration in vitro and angiogenesis in vivo. J. Cell. Biochem. 106: 200–209, 2009.

KEY WORDS: HEPARANASE; HEPARAN-SULFATE PROTEOGLYCANS; MELANOMA; MIGRATION; ANGIOGENESIS

Enzymatic remodeling of heparan sulfate proteoglycans (HSPG) within the tumor microenvironment is emerging as an important mechanism for the dynamic regulation of tumorigenesis [Iozzo, 2001; Sanderson et al., 2005; Vreys and David, 2007]. HSPG are a family of glycoproteins that are distinguished by the covalent attachment of one or more heparan sulfate (HS) chains to their protein core. HS are directly involved with the angiogenic process by acting as co-receptors with angiogenic growth factors [Iozzo, 2001]. As the interface between tumor cells and host cells, HS mediate cellular interactions. HS also influence tumor metastasis to sites such as the brain by arbitrating interactions between cancer cells, platelets, endothelial cells, and host organ cells. Intact HS prevent metastasis by acting as a physical barrier in the extracellular matrix (ECM). Enzymes that cleave HS may release fragments that can either support or inhibit tumorigenesis [Elkin et al., 2001; Liu et al., 2002a].

Heparanase (HPSE) is the only mammalian endo-β-D-glucuronidase which cleaves HS at specific intrachain sites, resulting in fragments of appreciable size (10–20 sugar units) [Ilan et al., 2006]. Numerous in vitro and in vivo studies have asserted a role for HPSE in tumor invasion and metastasis [Nakajima et al., 1988; Hulett et al., 1999; Vlodavsky et al., 1999; Uno et al., 2001; Edovitsky et al., 2004; Roy et al., 2005; Ilan et al., 2006]. In addition, HPSE activity is upregulated in human cancers; a number of studies using clinical samples demonstrated a correlation between tumor malignancy and HPSE levels [Friedmann et al., 2000; Kim et al., 2002; Maxhimer et al., 2002; Tang et al., 2002; Kelly et al., 2003; Doweck et al., 2006]. In vivo animal studies have also indicated that changes in the fine structure of tumor-cell-surface insoluble HS or soluble HS in the ECM have profound effects on tumor-cell growth kinetics, angiogenesis and metastasis formation [Elkin et al., 2001; Liu et al., 2002a; Dai et al., 2005; Narita et al., 2006; Reiland et al., 2006;

Abbreviations used: DMEM/F12, Dulbecco's modified Eagle's medium/F-12; BM, basement membrane; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; ECM, extracellular matrix; FGF2, fibroblast growth factor-2; Hep III, heparitinase III; HPSE, heparanase; HS, heparan sulfate; HSPG, heparan sulfate proteoglycans; P/S, penicillin/streptomycin; VEGF165, vascular endothelial growth factor isoform 165; VEGFR, VEGF receptor.

Grant sponsor: NIH; Grant numbers: 5R0-1 CA86832, 1R21 CA 103955.

*Correspondence to: Dr. Prof. Dario Marchetti, Department of Pathology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. E-mail: marchett@bcm.edu

Received 26 September 2008; Accepted 31 October 2008 • DOI 10.1002/jcb.22005 • 2008 Wiley-Liss, Inc.

Published online 29 December 2008 in Wiley InterScience (www.interscience.wiley.com).

Xu et al., 2007]. Depending upon HS composition and/or sequence involved in the process of tumorigenesis, they can either act as pro-tumorigenic, or anti-tumorigenic agents [Elkin et al., 2001; Liu et al., 2002a; Reiland et al., 2006].

Soluble or ECM HS can also differentially regulate fibroblast growth factor-2 (FGF2) binding and signaling leading to modification of angiogenesis of brain-metastatic melanoma cells depending upon its concentration [Reiland et al., 2006]. Amongst additional signaling molecules, some of HS-binding growth factors important for angiogenesis and tumor development are vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), transforming growth factor-β (TGF-β), and platelet-derived growth factor (PDGF) [Sasisekharan et al., 2002]. VEGF or vascular permeability factor (VPF) [Senger et al., 1983] was initially described as a highly specific mitogen for endothelial cells [Leung et al., 1989] and as a potent inducing agent for angiogenesis and vasculogenesis in a variety of physiological and pathological conditions [Ranieri et al., 2006]. Inhibition of VEGF signaling by VEGF antagonists, antisense VEGF, or dominant negative VEGF receptor (VEGFR) impaired tumorigenesis in vivo [Kim et al., 1993; Yano et al., 2000]. The VEGF family of proteins includes VEGF A through E and placenta growth factor [Ranieri et al., 2006]. VEGF A is the most studied and one of its splice variants, vascular endothelial growth factor isoform 165 (VEGF165), is the most potent and widely expressed isoform known [Plouet et al., 1997]. VEGF165 is secreted in the ECM as a disulfide-linked homodimer with two identical heparin-binding sites. HS, by binding to VEGF, regulate the diffusion, half-life, and affinity of VEGF165 to its cognate receptors [Gallagher, 2001; Iozzo, 2001].

We have shown previously that HPSE treatment or HPSE-generated bovine kidney HS products increase FGF2 binding and signaling in melanoma cells in vitro and FGF2-mediated angiogenesis in vivo. In this report, we have extended our study using cell surface HS from murine brain-metastatic melanoma cells (B16B15b) to investigate their effect on melanoma biology in respect to VEGF signaling. We sought to assess the role of HPSE-degraded cell-surface HS in VEGF-mediated activity in brain-metastatic melanoma cells since VEGF is known to be essential for brain metastasis in melanoma [Yano et al., 2000]. We hypothesized that HPSE contributes to melanoma metastasis by generating bioactive HS from the cell-surface that stimulate biological activities associated with the metastatic cascade. We also examined if these fragments could differentially affect VEGF165-mediated biological activities of melanoma and endothelioma. Here, we demonstrate that the isolated cell-surface HS stimulate in vitro migration but not proliferation of melanoma cells (B16B15b). Furthermore, they also promote in vivo angiogenesis by Matrigel™ plug assays. Interestingly, VEGF165 does not affect melanoma migration or angiogenesis alone or together with the cell-surface HS in these experiments. Finally, melanoma cell-surface HS do not stimulate in vitro migration of murine brain endothelioma cells (b.End3). Our results suggest that, in addition to remodeling the ECM and releasing ECM-based growth factors and chemokines, HPSE can contribute to aggressive phenotype of melanoma by releasing bioactive cell-surface HS which in turn stimulate melanoma cell migration and angiogenesis.

EXPERIMENTAL PROCEDURES

MATERIALS

Heparan sulfate from bovine kidney was purchased from Sigma Chemical Company (St. Louis, MO). Heparin-lyase III (heparitinase, EC 4.2.2.8) from *Flavobacterium heparinium* was obtained from Seikagaku America (Falmouth, MA). DMEM and Ham's F-12 nutrient medium and trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco BRL (Grand Island, New York, NY), and FBS from Hyclone Laboratories (Logan, UT). Reduced-growth factor Matrigel™ was obtained from BD Biosciences Discovery Labware (Bedford, MA). All other chemicals used were reagent grade or better.

CELLS AND TISSUE CULTURE CONDITIONS

Early-passage, *Mycoplasma*-negative, murine melanoma (B16B15b) cells with high brain-metastatic capabilities [Marchetti, 1997; Roy et al., 2005] were maintained as monolayer cultures in a 1:1 (v/v) mixture of Dulbecco's modified Eagle's medium/F-12 (DMEM/F12) supplemented with 5% (v/v) fetal bovine serum. Murine brain endothelioma cells (b.End3) [Montesano et al., 1990] were passaged in DMEM/F12 supplemented with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 10% (v/v) fetal bovine serum. Cells were maintained at 37°C in a humidified 5% CO_2/95% air (v/v) atmosphere and passaged using 2 mM EDTA (B16B15b) or trypsin-EDTA (b.End3) before reaching confluence.

For enzymatic treatment with HPSE, cells were washed three times in DMEM/F-12 containing 0.1% (w/v) bovine serum albumin (BSA), penicillin (100 U/ml), and streptomycin (100 μg/ml), then incubated with indicated concentrations (0–10 μg/ml) of recombinant HPSE in 50 mM HEPES-buffered DMEM/F-12 (pH 6.8) containing penicillin (100 U/ml) and streptomycin (100 μg/ml) for 18 h at 37°C in a shaker incubator at 50 rpm.

HPSE ISOLATION AND ACTIVITY

Recombinant human HPSE was purified as previously described [McKenzie et al., 2003; Reiland et al., 2006]. Briefly, Sf9 insect cells, transfected with baculovirus transfer vectors containing HPSE subunits, were grown in SF900II serum-free medium (Gibco BRL) 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 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 TBS, and 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 activity (Heparan Sulfate Degrading Enzyme Assay kit; Takara Mirus, Madison, WI) [McKenzie et al., 2003; Reiland et al., 2006]. HPSE eluted at 0.67 M NaCl, as expected [McKenzie et al., 2003; Reiland et al., 2006].

FLOW CYTOMETRIC ANALYSIS

Degradation of cell-surface HS was confirmed by flow cytometric analyses. Briefly, B16B15b metastatic melanoma cells were treated

with different concentrations of HPSE (0–10 μg/ml) for 18 h at 37°C using a shaker incubator. The medium was removed at the end of treatment and cells were collected by PBS–EDTA. Cells (5×10^5) were then incubated with HS mAb 10E4 (Seikagaku America) followed by incubations with super sensitive biotin-goat anti-mouse IgM (BioGenex, San Ramon, CA) and PE streptavidin (Molecular Probes, Eugene, OR), respectively. Cells were then fixed in 200 μl cold 1% (v/v) paraformaldehyde and stored at 4°C until analysis. Samples were analyzed for cell-surface HS staining using FACScan flow cytometer. Data were analyzed with WinMDI. Appropriate control samples without the primary and/or the secondary antibody (anti-IgM) were run to subtract background staining.

ISOLATION OF CELL SURFACE HS

B16B15b metastatic melanoma cells were treated with or without HPSE (10 μg/ml) overnight at 37°C using a shaker incubator. Conditioned medium was collected and pH was adjusted to 6.0. The medium was then centrifuged at 5,000 rpm for 10 min, filtered through 0.22 μm filters, and boiled for 10 min. The medium was incubated with DEAE-Sephacel (Sigma) for 18 h and subsequently poured onto the column. HS fragments bound to DEAE were washed with 0.1 M sodium phosphate and 0.15 M NaCl, pH 6.0. The fragments were gradient eluted with 0.5, 1.0, and 2.0 M NaCl in 0.1 M sodium phosphate buffer, pH 6.0. The fragments were then concentrated and buffer-exchanged into ultra-pure water by application to a Centricon filter (Millipore Corporation, Bedford, MA). Finally, isolated fragments were treated with pronase (Sigma) [Marchetti et al., 1997] to remove protein core from isolated HS.

HPSE-degraded HS used in biological experiments was analyzed by separating HS on a Criterion 4–20% TBE gel (Bio-Rad Laboratories, Hercules, CA) for 20 min at 60 mA. Bands were visualized with alcian blue 8GX (Sigma–Aldrich) followed by silver staining (Pierce Endogen, Rockford, IL) [Pervin et al., 1995]. Densitometric analyses were performed using a Versadoc imaging system (Bio-Rad Laboratories) to determine profiles leading edge. Non-treated commercial HS from bovine kidney were electrophoresed at various concentrations to obtain quantitative analysis.

WOUND HEALING ASSAY

Migratory properties of melanoma cells were analyzed by a standard wound healing assay. Briefly, cells were plated in 12-well plates at a high density and allowed to grow to confluence. Cells were washed three times in DMEM/F-12 containing 0.1% (w/v) BSA, penicillin (100 U/ml), and streptomycin (100 μg/ml) and then incubated with the same medium for 1 h. Using a sterile 100 μl tip, a single scratch was made through the middle of each well. The medium was subsequently removed and wells were rinsed three times with DMEM/F-12 containing 0.1% (w/v) BSA and penicillin/streptomycin to remove detached cells. Cell-surface HS (1 ng/ml) or recombinant VEGF165 (10–50 ng/ml) were added to cells in DMEM/F-12 containing 0.1% (w/v) BSA, 4 mM HEPES, penicillin (100 U/ml), and streptomycin (100 μg/ml) for 8 h at 37°C in a humidified 5% CO_2/95% air (v/v) atmosphere. Photomicrographs were taken at 0 h (T_0) and at the end of the experiment (T_8) using identical conditions to calculate percent relative gap closure. Relative gap closure was measured as $[1 - (T_8/T_0)]$. Migration assays for endothelioma were incubated for 24 h (T_{24}).

PROLIFERATION ASSAY

Proliferation of melanoma cells were assayed by using alamarBlue™ (BioSource International, Camarillo, CA) [Sinnappah-Kang et al., 2005], a non-toxic dye which monitors the reducing environment of the proliferating cell, as per the manufacturer's instructions. Briefly, 1×10^4 cells/ml were plated into 24-well plates and incubated for 24 h. At the start of the proliferation assay, cells were washed three times in DMEM/F-12 containing 0.1% (w/v) BSA and penicillin (100 U/ml) and streptomycin (100 μg/ml), and then incubated with the same medium for 1 h. Indicated concentrations of cell-surface HS or recombinant VEGF165 were added to cells in triplicates in DMEM/F-12 containing 0.1% (w/v) BSA, 4 mM HEPES, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified 5% CO_2/95% air (v/v) atmosphere. For indicated time points alamarBlue™ (10%, v/v) was added per well and were incubated for 4 h at 37°C in a humidified 5% CO_2/95% air (v/v) atmosphere. Cell proliferation was measured by monitoring the fluorescence of alamarBlue™ supplemented cell culture media at excitation and emission wavelengths of 540 and 630 nm, respectively. The greater is the percentage of reduction (fluorescent count), the higher is the proliferative activity.

IN VIVO ANGIOGENIC ASSAY

B16B15b cells were released with PBS–EDTA, washed two times in DMEM/F-12, and resuspended at 1×10^7 cells/ml in 50% (v/v) reduced-growth factor Matrigel™ (Becton Dickinson, Labware, Bedford, MA) in DMEM/F-12 at 4°C. HS fragments and VEGF165 were added accordingly. Cells (2×10^6) were injected using a 25-gauge needle to the left and right abdominal subcutaneous tissue of female C57BL6 (Harlan Teklan, Madison, WI) mice ($n = 6–9$). B16B15b cells, in reduced-growth factor Matrigel along with HPSE-degraded melanoma cell-surface HS or without, as mock control, were injected into the right (with VEGF) and left (without VEGF) abdominal subcutaneous tissue of female C57BL6 mice ($n = 6–9$). Animals were divided in three groups in a split-plot arrangement. Group A received HPSE-treated HS with VEGF (right) or without VEGF (left). Group B received HPSE-treated HS that were further treated with heparitinase III (Hep III) to cleave them into inactive disaccharide fragments with VEGF (right) or without VEGF (left). Group C received melanoma cells in mock-buffer with VEGF (right) or without VEGF (left). Mice were sacrificed on the 10th day; tumors were excised, fixed in 10% (v/v) formalin, and embedded in paraffin. Tumor sections (7 μm thick) were then stained with hematoxylene and eosin (H&E) and examined under the microscope. Blood vessel density was assessed by counting vessels within the tumor region in five sections in each tumor. Tumor sections were photographed using Olympus DP70 camera, Olympus BX45 microscope and saved in JPEG format using DP Manager (Olympus America, Inc., Center Valley, PA). Tumor areas were measured by counting pixels on ImageJ software (NIH). Pixel counts were converted to mm^2 to present the number of vessels per unit area. Statistical analyses were done using SAS (Version 9.1.3) in an analysis of variance in a split-plot arrangement of treatments. Main plot effects included group

and Animal Id within group; subplot effects included Side and Group × Side interaction. Pairwise comparisons of main effects were conducted with Tukey's HSD test. When appropriate, interaction effect comparisons were performed with *t*-tests of least-square means. All comparisons were considered significant at $P < 0.05$. Prior to analysis, the data were natural log-transformed to stabilize variance terms.

RESULTS

REMOVAL AND ISOLATION OF CELL-SURFACE HS BY HPSE TREATMENT

Highly brain-metastatic B16B15b melanoma cells were chosen as a source of HS since they possess high HSPG expression on the cell-surface [Reiland et al., 2004]. The extent of HS degradation by HPSE was assessed by detection of cell-surface HS on FACS analysis. Detectable reduction in HS expression levels was seen with as low as 5.0 ng/ml HPSE compared to no HPSE treatment (data not shown). To optimize isolation of cell-surface HS, when cells were treated with higher HPSE concentrations (100–10,000 ng/ml), a dose-dependent decrease in cell-surface HS expression was observed (Fig. 1A).

Conditioned medium following HPSE treatment (10 μg/ml) from B16B15b melanoma cells was collected and HS were isolated by ion-exchange column chromatography. Assessment of HPSE-mediated HS degradation was determined by gel electrophoresis of isolated fragments (Fig. 1B). Since the isolated HS were a heterogeneous mixture of oligosaccharides due to HPSE digestion, it migrated as a broad band during gel electrophoresis (Fig. 1B). The

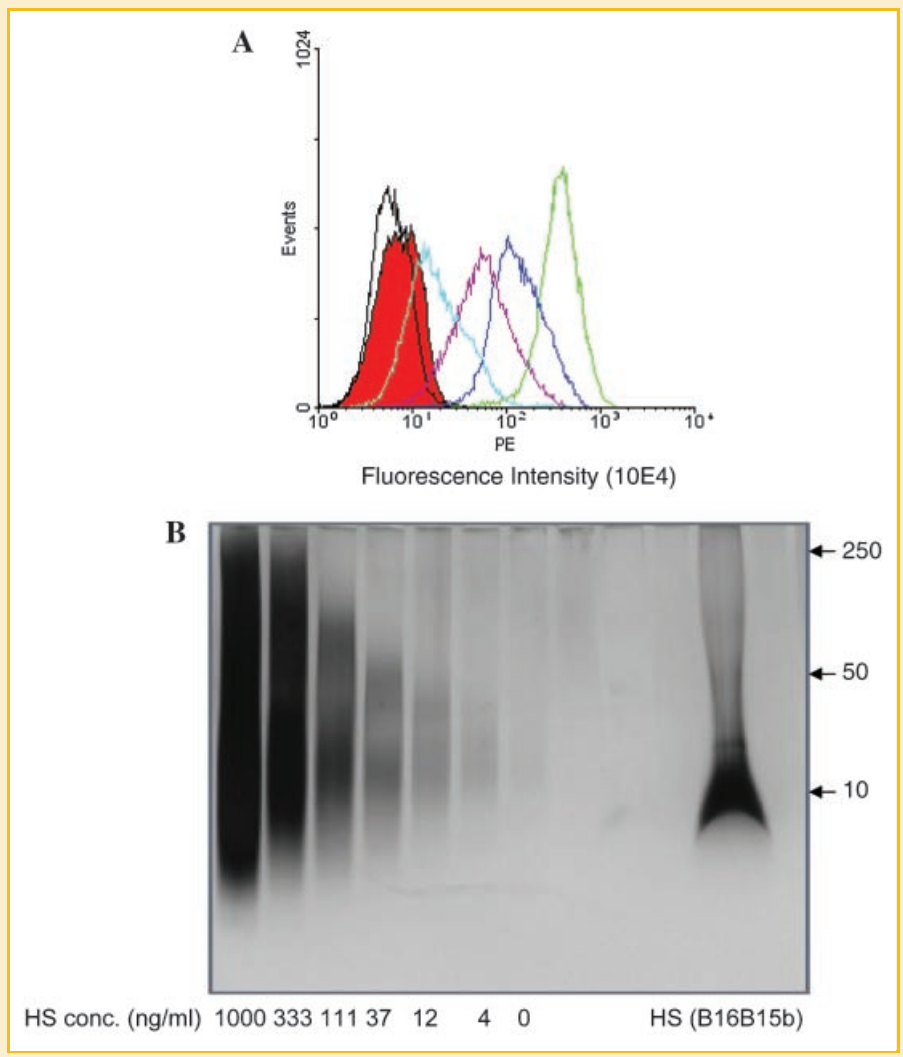

Fig. 1. Dose-dependent reduction of cell surface HSGAG with HPSE. A: Murine B16B15b melanoma cells were treated with 0–10,000 ng/ml HPSE. Cell surface HS level was detected by flow cytometry using mAb10E4. HPSE removes cell surface HSGAG in a dose-dependent manner. green, 0 ng/ml HPSE; blue, 100 ng/ml HPSE; purple, 1,000 ng/ml HPSE; light blue, 10,000 ng/ml HPSE. Appropriate controls were run to account for background staining. black, no primary antibody control; solid red, no primary and no secondary antibody control. B: HPSE-degraded cell surface HSGAG profile on silver stain. As expected, HPSE digestion generated HS fragments of about 10 kDa. Various concentrations of untreated HS were run to generate a standard curve for determination of concentration of HSGAG after densitometric analysis. Numbers on the right-hand side of figure refer to molecular weight (MW) standards (kDa).

leading edge of HS profiles was determined after densitometric analysis and concentration was determined by generating a curve with known standards (Fig. 1B).

EFFECTS OF HPSE-DEGRADED HS ON ENDOTHELIOMA IN VITRO

Angiogenesis is an important step in solid tumor growth beyond a certain dimension (0.2–2.0 mm or about 10^5–10^6 cells) that requires formation of new blood vessels from the preexisting vascular network [Folkman, 1971]. Endothelial cells migrate and proliferate during angiogenesis and are influenced by the tumor microenvironment including heparin/HS-binding growth factors secreted by the tumors such as FGF2 and VEGF [Carmeliet, 2000]. Therefore, we studied effects of HPSE-degraded HS in an endothelial system. We investigated changes in migratory properties in murine brain endothelioma cell line b.End3, since migration is a critical event in angiogenesis [Carmeliet, 2000].

To study how exogenous addition of HS will influence endothelioma (b.End3) biological activity, we added HPSE-digested melanoma cell surface HS to serum-free endothelioma medium. HS did not have any effect on endothelioma cell migration (Fig. 2). We also used VEGF165, a known mitogenic factor for endothelial cells, which stimulated endothelioma cell migration compared to no VEGF treatment ($P < 0.05$, Fig. 2). Moreover, addition of melanoma cell surface HS to VEGF165 treatment did not augment this response (Fig. 2).

HPSE-DEGRADED CELL-SURFACE HS MODULATES MELANOMA CELL MIGRATION

To directly test whether the B16B15b cell-surface HS were biologically active, we tested their effects on melanoma cell migration. B16B15b cells possess an aggressive migratory behavior, express VEGF receptors, and respond to VEGF. Interestingly, when HS (1 ng/ml) were added externally, there was a 30% up to a twofold increase in cell migration compared to no treatment ($P < 0.05$, Fig. 3). Addition of VEGF165 (0–100 ng/ml) did not augment this effect compared to no VEGF165 control (data not shown). Interestingly, VEGF165 did not effect migration even when added along with HS compared to HS alone (Fig. 3). One possibility could be that melanoma cells tested secrete autocrine growth factors [Menter et al., 1994] even after serum starvation, and thus do not respond to exogenously added stimuli. This was unexpected since VEGF165 is known to require HS to exert its biological effects. However, changes in cell migratory properties following addition of melanoma HS isolated by HPSE digestion suggested that the HPSE-degraded HS fragments are bioactive and possess tumor stimulatory activity.

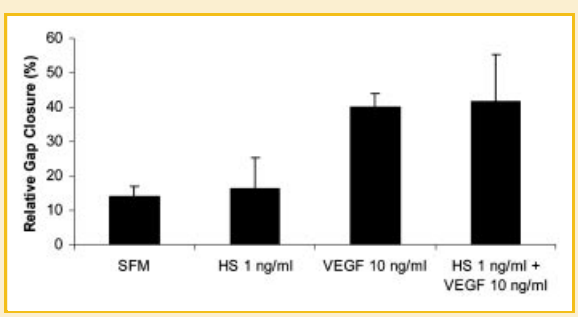

Fig. 2. Endothelioma cell migration is not influenced by HPSE-degraded HS but VEGF stimulates wound healing. To study how exogenous addition of HS will influence endothelioma (b.End3) biological activity, we added HPSE-digested melanoma cell-surface HS to serum-free endothelioma medium. HS treatment did not stimulate endothelioma cell migration while VEGF did ($P < 0.05$), as expected. HS, when added along with VEGF, did not augment VEGF response.

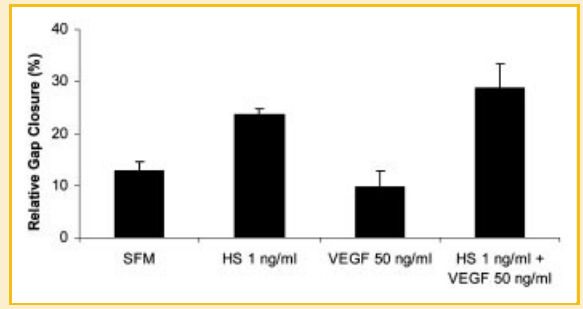

Fig. 3. HPSE-degraded cell surface HS modulate melanoma cell migration. When HS (1 ng/ml) were added externally to melanoma cells in wound healing assays, there was increased migration compared to control ($P < 0.05$). Addition of VEGF (50 ng/ml) did not affect migration with or without HS.

HPSE-DEGRADED CELL-SURFACE HS DOES NOT INFLUENCE MELANOMA CELL PROLIFERATION

We next explored effects of B16B15b cell-surface HS on melanoma cell proliferation to test if similar conditions used in our wound healing assays also affect cell proliferation. Melanoma cell proliferation was assayed by alamarBlue™ (see "Experimental Procedures" Section), cell proliferation was monitored every 24 h for 72 h. The basal cell proliferation rate is high in these cells; we did not observe any change in proliferative properties of the cells either by HS or by VEGF treatment over a period of 72 h (Fig. 4). Thus, the

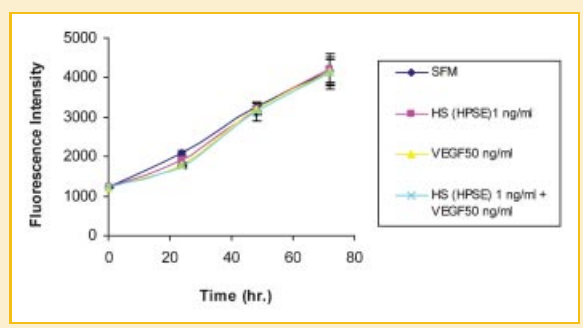

Fig. 4. HPSE-degraded cell-surface HSGAG does not influence melanoma cell proliferation. Proliferation of melanoma cells were assayed by alamarBlue™, a non-toxic dye that monitors the reducing environment of the proliferating cell. Melanoma cell proliferation was monitored every 24 h for 72 h. Exogenous addition of VEGF (50 ng/ml) or melanoma cell surface HS (1 ng/ml) isolated by HPSE treatment did not influence cell proliferation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

exogenous addition of melanoma cell-surface HS isolated by HPSE treatment influences melanoma cell migration without affecting proliferation.

HPSE-DEGRADED CELL-SURFACE HS PROMOTES IN VIVO ANGIOGENESIS

To investigate effects of HPSE-derived cell-surface HS on in vivo angiogenesis, Matrigel plug assays were performed. B16B15b cells, in reduced-growth factor Matrigel along with HPSE-degraded melanoma cell-surface HS (1 ng/ml) or without, as mock control, were injected into the right (with 50 ng/ml VEGF) and left (without VEGF) abdominal subcutaneous tissue of female C57BL6 mice ($n = 6–9$). Mice were sacrificed on the 10th day post-injection and Matrigel plugs were removed. Sections (7 μm thick) were then H&E-stained to examine for blood vessel formation. Blood vessel density was assessed by counting vessels within the Matrigel plug region in five sections in each plug.

HPSE-treated cell surface HS induced a significant increase in intratumor blood vessel formation (Fig. 5A,B) in animals in group A compared to mock (group C) or Hep III treatment of HPSE-derived cell-surface HS (group B; $P < 0.0001$). Interestingly, the increased numbers of tumor vessel formation were VEGF-independent. Presence of VEGF did not affect angiogenesis in all three groups ($P = 0.2–0.8$). Notably, the absence of blood vessels inside the tumors led to areas of necrosis due to lack of nutrients and oxygen (Fig. 5A). Importantly, the Matrigel plugs in all groups seemed to be approximately the same size and this observation was confirmed following excision and weighing of the plugs (not shown). Therefore, the differences in vascularity between the mock control and/or Hep III control versus the HPSE-generated cell-surface HS tumors are not due to differences in tumor size, rather it is due to the tumor promoting effects of cell-surface HS fragments on tumor microenvironment.

DISCUSSION

In the present study, we have investigated roles of HPSE-degraded cell-surface HS in melanoma tumorigenesis and possible effects on host endothelial system. Our findings suggest that melanoma cell-surface HS isolated by HPSE treatment promotes (1) melanoma migration and (2) angiogenesis independent of VEGF activity. These results also provide evidence that, in addition to remodeling the ECM and releasing growth factors and chemokines, HPSE contributes to the aggressive phenotype of melanoma by releasing bioactive HS which stimulate melanoma tumorigenesis.

HSPG are recognized as key cell-surface/ECM active biological modulators [Elkin et al., 2001; Iozzo, 2001; Liu et al., 2002a,b; Sasisekharan et al., 2002]. Their degradation at the level of HS chains by glycosidases has significant regulatory consequences in cancer metastasis [Esko and Selleck, 2002]. HS present on tumor cells also contain bioactive sequences that may affect tumor-cell phenotype in relation to cell growth and metastasis [Liu et al., 2002a,b; Dai et al., 2005; Narita et al., 2006]. It has been established that growth factor binding to HS which leads to mitogenic activity takes place only when definite structural features are present within the HS chains, such as, sulfation at specific positions within a disaccharide (N, 2-O, 3-O, 6-O) by the enzymes mediating HS synthesis within the Golgi apparatus [Esko and Selleck, 2002]. On the other hand, it has also been shown that besides the modification that occurs in the Golgi during its synthesis and expression, HS can also be structurally and functionally modulated within the extracellular compartment. The two families of mammalian enzymes currently known to modify HS are the endosulfatases (Hsulf-1 and -2) which remove 6-O sulfation on the HS [Dai et al., 2005; Narita et al., 2006] and HPSE, which cleaves HS into small, biologically active fragments [Parish et al., 1987; Hulett et al., 1999; Kussie et al., 1999; Vlodavsky et al., 1999; Dempsey et al., 2000; Elkin et al., 2001; Reiland et al., 2004; Ilan et al., 2006]. HPSE has also been shown to promote shedding of cell-surface syndecan-1 and modify tumorigenesis [Mahtouk et al., 2007; Yang et al., 2007].

Elevated levels of HPSE are known to be associated with brain-metastatic melanoma [Uno et al., 2001; Edovitsky et al., 2004; Roy et al., 2005]. The enzymatic activity of HPSE is characterized by specific intrachain HS cleavage of glycosidic bonds with a hydrolase (but not eliminase) type of action that facilitates the release of several protein modulators of cell function, including migration, adhesion, inflammation, angiogenesis, embryogenesis, and metastatic invasion [Elkin et al., 2001; Ilan et al., 2006; McKenzie, 2007; Vreys and David, 2007]. When over-expressed, HPSE increases tumor cell invasiveness in vitro and in vivo settings [Uno et al., 2001; Roy et al., 2005]; conversely, a downregulation of HPSE by anti-sense or siRNA methodologies decreases tumorigenesis [Uno et al., 2001; Edovitsky et al., 2004; Roy et al., 2005]. However, at higher concentration HPSE can also inhibit tumorigenesis possibly by extensive remodeling of cell-surface HS that interferes with growth factor binding and signaling leading to subsequent inhibition of biological effects [Zetser et al., 2003; Reiland et al., 2006; Roy and Marchetti, unpublished observations].

Extensive evidence suggests that cellular function and phenotype are highly influenced by the composition and size of HS chains on HSPG [Elkin et al., 2001; Liu et al., 2002a,b; Sasisekharan et al., 2002]. A cell can respond to its microenvironment in markedly different ways by dynamically regulating HS structure on its cell-surface as insoluble HS and in the ECM as soluble HS [Elkin et al., 2001; Liu et al., 2002a,b; Sasisekharan et al., 2002; Reiland et al., 2006]. HSPG and HS chains are present on the surface of all eukaryotic cells, including tumor cells. This is valid also for cells that are important for tumor survival, for example, endothelial-cell junction surrounding a growing tumor, where HS can participate in the process of angiogenesis. This led us to believe that proangiogenic activity of HPSE could partly be due to generation of bioactive fragments by its enzymatic activity. We found that these fragments are indeed active and probably mediate their effects through melanoma autocrine/paracrine factors.

Highly brain-metastatic B16B15b melanoma cells were chosen as a source of HS since they express large amount of it on the cell-surface [Reiland et al., 2004]. The extent of HS degradation on B16B15b by HPSE was assessed by detecting cell-surface HS on FACS analysis. We were able to remove melanoma (B16B15b)

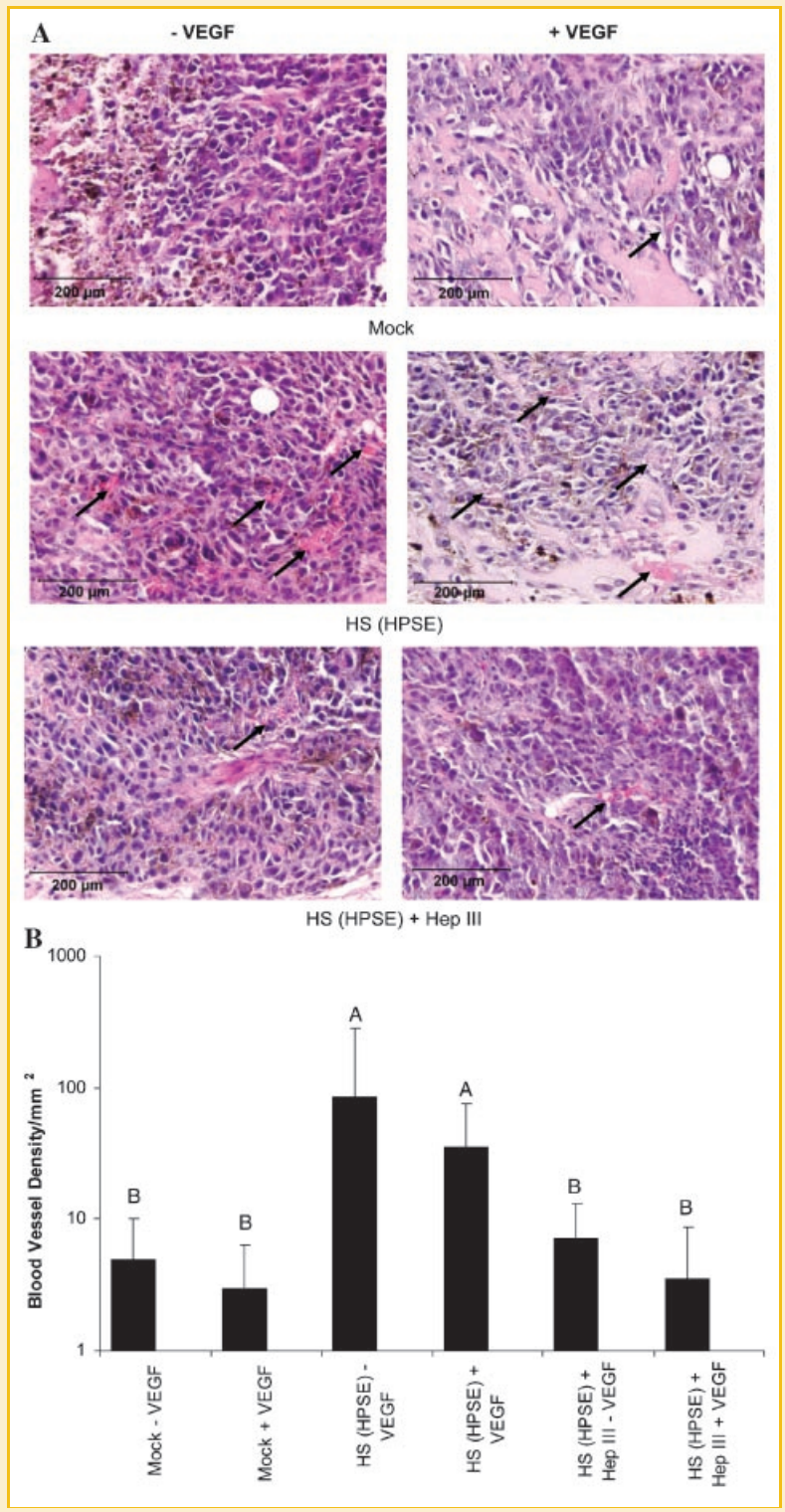

Fig. 5. HPSE-degraded cell surface HSGAG promotes angiogenesis in vivo. Blood vessel density was assessed by counting vessels within the tumor region in five different sections in each tumor. Tumor sections were photographed using Olympus DP70 camera, Olympus BX45 microscope and saved as JEPG format using DP Manager (Olympus). Tumor areas were measured by counting pixels on ImageJ software (NIH). Pixel counts were converted to mm^2 to present the number of vessels per unit area. Statistical analyses were done using SAS (Version 9.1.3) in an analysis of variance in a split-plot arrangement of treatments. A: Representative tumor sections from each treatment group (H&E). HPSE-treated cell surface HSGAG induced a significant increase in intratumor blood vessel (arrow) formation in animals compared to mock or Hep III treatment of HPSE-treated cell surface HSGAG ($P < 0.0001$). Hep III treatment renders the HPSE-degraded fragments inactive, hence abolishes their biological activity. Presence of VEGF did not affect angiogenesis in all three groups ($P = 0.2–0.8$). Notably, inside the tumors, absence of blood vessels, thereby lack of nutrition and oxygen led to areas of necrosis. B: Bar graph representation of mean blood vessel density with standard deviation plotted on a log scale. Bars with the same letter are not significantly different from each other.

cell-surface HS with HPSE treatment in a dose-dependent manner as indicated by the leftward shift in the profile on the *x*-axis (Fig. 1A). The associated protein core due to shed syndecan-1 by direct action of HPSE [Mahtouk et al., 2007; Yang et al., 2007] and other possible protein contamination were removed by pronase digestion during the isolation process. As expected, HPSE digestion generated HS fragments of about 10 kDa (Fig. 1B).

To directly demonstrate whether the B16B15b cell-surface HS were biologically active, we tested their effects on melanoma and endothelioma biological activity. We reasoned that because VEGF is a HS-binding growth factor and is essential for brain metastasis formation [Yano et al., 2000], these fragments would participate in VEGF-mediated activities. Isoforms of VEGF can bind VEGF receptors in the absence of HS, but this interaction is enhanced by cellular or exogenous heparin/HS suggesting that heparin/HS on HSPG regulate the interaction of VEGF-VEGF receptor and subsequent biological activity [Terman et al., 1994; Schlessinger et al., 1995; Gitay-Goren et al., 1996; Ferrara et al., 2003; Lake et al., 2006]. Interestingly, in our melanoma cell system, the presence of VEGF did not influence the biological activities of the HPSE-degraded melanoma cell-surface HS including migration (Fig. 3) and proliferation (Fig. 4) of melanoma in vitro, and angiogenesis in vivo (Fig. 5). We also investigated effects on melanoma migration by adding FGF2, platelet-derived growth factor (PDGF) and interleukin-8 (IL-8) but no difference in cell migration was observed compared to no growth factor control (data not shown). A possibility could be that VEGF bound to endogenous cell-surface HS and/or because the melanoma cells already have autocrine production of VEGF, we did not observe an added effect with exogenously added growth factor [Menter et al., 1994]. A recent report by Robinson et al. [2006] demonstrated that VEGF requires highly sulfated sites on the HS for binding. According to this study, these sites were exposed by enzymatic action of K5-lyase on HS and it retained significant VEGF165 affinity; in contrast, cleavage of HS by heparinases or HPSE severely reduced VEGF165 binding [Robinson et al., 2006]. This may explain the reason we failed to observe any biological effect with exogenously added VEGF along with HPSE-derived HS in melanoma cell activity. However, melanoma cell-surface HS were able to stimulate melanoma migration (Fig. 3) and angiogenesis (Fig. 5) compared to the controls suggesting that these fragments are tumorigenic although they do not affect melanoma cell proliferation (Fig. 4). The enhanced angiogenesis by these fragments were possibly due to signaling by some other heparin-binding growth factor(s) which could either stimulate tumorigenesis or alternatively, could abolish tumor inhibitory signal. In addition, there were no significant differences observed in tumor weight in all treatment groups, hence the increased vascularity seen with the HPSE-generated cell-surface HS tumors were due to its effects on the tumor microenvironment. These findings are further strengthened by the fact that, following Hep III-mediated digestion into smaller fragments, HPSE-degraded HS lose their proangiogenic properties (Fig. 5).

The HPSE-degraded HS did not have any effects on in vitro b.End3 endothelioma cell migration (Fig. 2), or signaling (data not shown) which could be due to tissue-specific HS structural differences present between the systems [Esko and Lindahl, 2001]. Even though, the same sets of disaccharides are present in most tissues, their relative content varies quantitatively in terms of sulfation or epimerization pattern [Esko and Lindahl, 2001]. Attempts to remove endothelioma cell-surface HS by enzymatic degradation of Hep III or HPSE did not alter response to growth factor in this system (data not shown). This could be due to the fact that removal of cell-surface HS was incomplete and remaining quantities of cell-surface HS were sufficient to arbitrate growth factor-mediated signaling which is known to occur [Krufka et al., 1996]. Nonetheless, the in vivo induction of angiogenesis by the melanoma cell HS fragments could also be due to availability of additional support to the endothelial cells directly or indirectly from the tumor cells.

Remodeling of the ECM and basement membrane (BM) is vital for a normal embryonic development, wound healing and tumorigenesis. During tumor progression, this turnover is highly controlled and involves the coordinated action of proteases and endoglycosidases [Sanderson et al., 2005; Vreys and David, 2007]. This process not only contributes to angiogenesis and tumor invasion by altering the integrity of the BM/ECM, but also results in the release of HS-binding molecules such as chemokines and proangiogenic growth factors, initiating numerous downstream signaling cascades. While large families of proteases (matrix metalloproteases, aspartic, cysteine, and serine proteases) mediate the cleavage of protein components of the BM/ECM, cleavage of the HS side chains is performed by a limited set of enzymes, notably HPSE [Sanderson et al., 2005; Vreys and David, 2007]. Characterization of these HPSE-degraded melanoma cell-surface HS would potentially be useful. Devising a method that would isolate individual oligosaccharides is required to test for pro-angiogenic/pro-tumorigenic properties of the fragments. Moreover, the design of novel agents targeted against these HS fragments can be an important addition to developing polysaccharide based anti-tumor therapy in melanoma.

ACKNOWLEDGMENTS

We thank Dr. William Henk and Mr. Gregory McCormick for assistance with microscopy, Dr. Jane Reiland for helpful discussions, Dr. Daniel Paulsen for pathology reviews, and Michel T Kearney for statistical analyses (LSU-Baton Rouge). This work was supported by grants from NIH (5R0-1 CA86832 and 1R21 CA 103955 to D.M.).

REFERENCES

Carmeliet P. 2000. Mechanisms of angiogenesis and arteriogenesis. Nat Med 6:389–395.

Dai Y, Yang Y, MacLeod V, Yue X, Rapraeger AC, Shriver Z, Venkataraman G, Sasisekharan R, Sanderson RD. 2005. HSulf-1 and HSulf-2 are potent inhibitors of myeloma tumor growth in vivo. J Biol Chem 280:40066–40073.

Dempsey LA, Brunn GJ, Platt JL. 2000. Heparanase, a potential regulator of cell-matrix interactions. Trends Biochem Sci 25:349–351.

Doweck I, Kaplan-Cohen V, Naroditsky I, Sabo E, Ilan N, Vlodavsky I. 2006. Heparanase localization and expression by head and neck cancer: Correlation with tumor progression and patient survival. Neoplasia 8:1055–1061.

Edovitsky E, Elkin M, Zcharia E, Peretz T, Vlodavsky I. 2004. Heparanase gene silencing, tumor invasiveness, angiogenesis, and metastasis. J Natl Cancer Inst 96:1219–1230.

Elkin M, Ilan M, Ishai-Michaeli R, Friedmann Y, Pappo O, Pecker I, Vlodavsky I. 2001. Heparanase as mediator of angiogenesis: Mode of action. FASEB J 15:1661–1663.

Esko JD, Lindahl U. 2001. Molecular diversity of heparan sulfate. J Clin Invest 108:169–173.

Esko JD, Selleck SB. 2002. Order out of chaos: Assembly of ligand binding sites in heparan sulfate. Ann Rev Biochem 71:435–471.

Ferrara N, Gerber H-P, LeCouter J. 2003. The biology of VEGF and its receptors. Nat Med 9:669–676.

Folkman J. 1971. Tumor angiogenesis: Therapeutic implications. N Engl J Med 285:1182–1186.

Friedmann Y, Vlodavsky I, Aingorn H, Aviv A, Peretz T, Pecker I, Pappo O. 2000. Expression of heparanase in normal, dysplastic, and neoplastic human colonic mucosa and stroma: Evidence for Its role in colonic tumorigenesis. Am J Pathol 157:1167–1175.

Gallagher JT. 2001. Heparan sulfate: Growth control with a restricted sequence menu. J Clin Invest 108:357–361.

Gitay-Goren H, Cohen T, Tessler S, Soker S, Gengrinovitch S, Rockwell P, Klagsbrun M, Levi BZ, Neufeld G. 1996. Selective binding of VEGF121 to one of the three vascular endothelial growth factor receptors of vascular endothelial cells. J Biol Chem 271:5519–5523.

Hulett MD, Freeman C, Hamdorf BJ, Baker RT, Harris MJ, Parish CR. 1999. Cloning of mammalian heparanase, an important enzyme in tumor invasion and metastasis. Nat Med 5:803–809.

Ilan N, Elkin M, Vlodavsky I. 2006. Regulation, function and clinical significance of heparanase in cancer metastasis and angiogenesis. Int J Biochem Cell Biol 38:2018–2039.

Iozzo RV. 2001. Heparan sulfate proteoglycans: Intricate molecules with intriguing functions. J Clin Invest 108:165–167.

Kelly T, Miao HQ, Yang Y, Navarro E, Kussie P, Huang Y, MacLeod V, Casciano J, Joseph L, Zhan F, Zangari M, Barlogie B, Shaughnessy J, Sanderson RD. 2003. High heparanase activity in multiple myeloma is associated with elevated microvessel density. Cancer Res 63:8749–8756.

Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N. 1993. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature 362:841–844.

Kim AW, Xu X, Hollinger EF, Gattuso P, Godellas CV, Prinz RA. 2002. Human heparanase-1 gene expression in pancreatic adenocarcinoma. J Gastrointest Surg 6:167–172.

Krufka A, Guimond S, Rapraeger AC. 1996. Two hierarchies of FGF-2 signaling in heparin: Mitogenic stimulation and high-affinity binding/receptor transphosphorylation. Biochemistry 35:11131–11141.

Kussie PH, Hulmes JD, Ludwig DL, Patel S, Navarro EC, Seddon AP, Giorgio NA, Bohlen P. 1999. Cloning and functional expression of a human heparanase gene. Biochem Biophys Res Commu 261:183–187.

Lake AC, Vassy R, Di Benedetto M, Lavigne D, Le Visage C, Perret GY, Letourneur D. 2006. Low molecular weight fucoidan increases VEGF165-induced endothelial cell migration by enhancing VEGF165 binding to VEGFR-2 and NRP1. J Biol Chem 281:37844–37852.

Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. 1989. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246: 1306–1309.

Liu D, Shriver Z, Venkataraman G, El Shabrawi Y, Sasisekharan R. 2002a. Tumor cell surface heparan sulfate as cryptic promoters or inhibitors of tumor growth and metastasis. Proc Natl Acad Sci USA 99:568–573.

Liu D, Shriver Z, Qi Y, Venkataraman G, Sasisekharan R. 2002b. Dynamic regulation of tumor growth and metastasis by heparan sulfate glycosaminoglycans. Semin Thromb Hemost 67–78.

Mahtouk K, Hose D, Raynaud P, Hundemer M, Jourdan M, Jourdan E, Pantesco V, Baudard M, De Vos J, Larroque M, Moehler T, Rossi J-F, Reme T, Goldschmidt H, Klein B. 2007. Heparanase influences expression and shedding of syndecan-1, and its expression by the bone marrow environment is a bad prognostic factor in multiple myeloma. Blood 109:4914–4923.

Marchetti D. 1997. Specific degradation of subendothelial matrix proteoglycans by brain-metastatic melanoma and brain endothelial cell heparanases. J Cell Physiol 172:334–342.

Marchetti D, Liu S, Spohn WC, Carson DD. 1997. Heparanase and a synthetic peptide of heparan sulfate-interacting protein recognize common sites on cell surface and extracellular matrix heparan sulfate. J Biol Chem 272: 15891–15897.

Maxhimer JB, Quiros RM, Stewart R, Dowlatshahi K, Gattuso P, Fan M, Prinz RA, Xu X. 2002. Heparanase-1 expression is associated with the metastatic potential of breast cancer. Surgery 132:326–333.

McKenzie EA. 2007. Heparanase: A target for drug discovery in cancer and inflammation. Br J Pharmacol 151:1–14.

McKenzie E, Young K, Hircock M, Bennett J, Bhaman M, Felix R, Turner P, Stamps A, McMillan D, Saville G, Ng S, Mason S, Snell D, Schofield D, Gong H, Townsend R, Gallagher J, Page M, Parekh R, Stubberfield C. 2003. Biochemical characterization of the active heterodimer form of human heparanase (Hpa1) protein expressed in insect cells. Biochem J 373:423–435.

Menter DG, Herrmann JL, Marchetti D, Nicolson GL. 1994. Involvement of neurotrophins and growth factors in brain metastasis formation. Invasion Metastasis 14:372–384.

Montesano R, Pepper MS, Mohle-Steinlein U, Risau W, Wagner EF, Orci L. 1990. Increased proteolytic activity is responsible for the aberrant morphogenetic behavior of endothelial cells expressing the middle T oncogene. Cell 62:435–445.

Nakajima M, Irimura T, Nicolson GL. 1988. Heparanases and tumor metastasis. J Cell Biochem 36:157–167.

Narita K, Staub J, Chien J, Meyer K, Bauer M, Friedl A, Ramakrishnan S, Shridhar V. 2006. HSulf-1 inhibits angiogenesis and tumorigenesis in vivo. Cancer Res 66:6025–6032.

Parish CR, Coombe DR, Jakobsen KB, Bennett FA, Underwood PA. 1987. Evidence that sulphated polysaccharides inhibit tumour metastasis by blocking tumour-cell-derived heparanases. Int J Cancer 40:511–518.

Pervin A, Gallo C, Jandik KA, Han XJ, Linhardt RJ. 1995. Preparation and structural characterization of large heparin-derived oligosaccharides. Glycobiology 5:83–95.

Plouet J, Moro F, Bertagnolli S, Coldeboeuf N, Mazarguil H, Clamens S, Bayard F. 1997. Extracellular cleavage of the vascular endothelial growth factor 189-amino acid form by urokinase is required for its mitogenic effect. J Biol Chem 272:13390–13396.

Ranieri G, Patruno R, Ruggieri E, Montemurro S, Valerio P, Ribatti D. 2006. Vascular endothelial growth factor (VEGF) as a target of bevacizumab in cancer: From the biology to the clinic. Curr Med Chem 13:1845–1857.

Reiland J, Sanderson RD, Waguespack M, Barker SA, Long R, Carson DD, Marchetti D. 2004. Heparanase degrades syndecan-1 and perlecan heparan sulfate: Functional implications for tumor cell invasion. J Biol Chem 279: 8047–8055.

Reiland J, Kempf D, Roy M, Denkins Y, Marchetti D. 2006. FGF2 binding, signaling, and angiogenesis are modulated by heparanase in metastatic melanoma cells. Neoplasia 8:596–606.

Robinson CJ, Mulloy B, Gallagher JT, Stringer SE. 2006. VEGF165-binding sites within heparan sulfate encompass two highly sulfated domains and can be liberated by K5 lyase. J Biol Chem 281:1731–1740.

Roy M, Reiland J, Murry BP, Chouljenko V, Kousoulas KG, Marchetti D. 2005. Antisense-mediated suppression of heparanase gene inhibits melanoma cell invasion. Neoplasia 7:253–262.

Sanderson RD, Yang. Y, Kelly T, MacLeod V, Dai Y, Theus A. 2005. Enzymatic remodeling of heparan sulfate proteoglycans within the tumor microenvironment: Growth regulation and the prospect of new cancer therapies. J Biol Chem 96:897–905.

Sasisekharan R, Shriver Z, Venkataraman G, Narayanasami U. 2002. Roles of heparan-sulphate glycosaminoglycans in cancer. Nat Rev Cancer 2:521–528.

Schlessinger J, Lax I, Lemmon M. 1995. Regulation of growth factor activation by proteoglycans: What is the role of the low affinity receptors? Cell 83:357–360.

Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. 1983. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 219:983–985.

Sinnappah-Kang ND, Kaiser AJ, Blust BE, Mrak RE, Marchetti D. 2005. Heparanase, TrkC and p75NTR: Their functional involvement in human medulloblastoma cell invasion. Int J Oncol 27:617–626.

Tang W, Nakamura Y, Tsujimoto M, Sato M, Wang X, Kurozumi K, Nakahara M, Nakao K, Nakamura M, Mori I, Kakudo K. 2002. Heparanase: A key enzyme in invasion and metastasis of gastric carcinoma. Mod Pathol 15:593–598.

Terman B, Khandke L, Dougher-Vermazan M, Maglione D, Lassam NJ, Gospodarowicz D, Persico MG, Bohlen P, Eisinger M. 1994. VEGF receptor subtypes KDR and FLT1 show different sensitivities to heparin and placenta growth factor. Growth Factors 11:187–195.

Uno F, Fujiwara T, Takata Y, Ohtani S, Katsuda K, Takaoka M, Ohkawa T, Naomoto Y, Nakajima M, Tanaka N. 2001. Antisense-mediated suppression of human heparanase gene expression inhibits pleural dissemination of human cancer cells. Cancer Res 61:7855–7860.

Vlodavsky I, Friedmann Y, Elkin M, Aingorn H, Atzmon R, Ishai-Michaeli R, Bitan M, Pappo O, Peretz T, Michal I, Spector L, Pecker I. 1999. Mammalian heparanase: Gene cloning, expression and function in tumor progression and metastasis. Nat Med 5:793–802.

Vreys V, David G. 2007. Mammalian heparanase: What is the message? J Cell Mol Med 11:427–452.

Xu X, Rao G, Quiros RM, Kim AW, Miao H-Q, Brunn GJ, Platt JL, Gattuso P, Prinz RA. 2007. In vivo and in vitro degradation of heparan sulfate (HS) proteoglycans by HPR1 in pancreatic adenocarcinomas: Loss of cell surface HS suppresses fibroblast growth factor 2-mediated cell signaling and proliferation. J Biol Chem 282:2363–2373.

Yang Y, MacLeod V, Miao H-Q, Theus A, Zhan F, Shaughnessy JD Jr, Sawyer J, Li J-P, Zcharia E, Vlodavsky I, Sanderson RD. 2007. Heparanase enhances syndecan-1 shedding: A novel mechanism for stimulation of tumor growth and metastasis. J Biol Chem 282:13326–13333.

Yano S, Shinohara H, Herbst RS, Kuniyasu H, Bucana CD, Ellis LM, Davis DW, McConkey DJ, Fidler IJ. 2000. Expression of vascular endothelial growth factor is necessary but not sufficient for production and growth of brain metastasis. Cancer Res 60:4959–4967.

Zetser A, Bashenko Y, Miao H-Q, Vlodavsky I, Ilan N. 2003. Heparanase affects adhesive and tumorigenic potential of human glioma cells. Cancer Res 63:7733–7741.