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Role of heparanase and heparanase-degraded heparan sulfate in brain-metastatic melanoma

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ROLE OF HEPARANASE AND HEPARANASE-DEGRADED HEPARAN SULFATE IN BRAIN-METASTATIC MELANOMA

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BSA……bovine serum albumin

DMEM……..Dulbecco's modified Eagle's medium

ECM…….……..…extracellular matrix

EDTA……..ethylenediaminetetraacetic acid

ELISA…….....enzyme-linked immunosorbent assay

FBS……..…….fetal bovine serum

FGF2…….fibroblast growth factor-2

FITC…….fluorescein isothiocyanate

FITC-HS……..FITC-labeled heparan sulfate

HPLC……...……high-speed gel permeation column chromatography

HPSE-1…….heparanase

HRP……..…horseradish peroxidase

HS……..heparan sulfate

HSGAG……..…heparan sulfate glycosaminoglycan chains

HSPG……...……...heparan sulfate proteoglycans

IgG……...immunoglobulin G

IgM…….immunoglobulin M

mAb……monoclonal antibody

PAb……polyclonal antibody

PBS……...phosphate-buffered saline

P/S…….penicillin/streptomycin

VEGF…….vascular endothelial growth factor

ABSTRACT

Cancer metastasis is a frequent manifestation of malignant melanoma progression. Successful invasion into distant organs by tumor cells must include attachment to microvessel endothelial cells, and degradation of extracellular matrix. Heparan sulfate proteoglycans are ubiquitous macromolecules associated with cell surface and extracellular matrix of a wide range of cells and tissues. Heparanase is an extracellular matrix degradative enzyme which degrades the heparan sulfate chains of heparan sulfate proteoglycans. To investigate effects of changes in *heparanase* gene expression in metastatic melanoma cells, we constructed adenoviral vectors containing the full-length human heparanase cDNA in both sense (Ad-S/hep) and anti-sense orientations (Ad-AS/hep). We demonstrated increased heparanase expression and activity in melanoma cell lines following Ad-S/hep infection by Western blot analyses and heparanase activity assay. Conversely, heparanase content was significantly inhibited following infection with Ad-AS/hep. Alteration of heparanase protein expression by these adenoviral constructs correlated with invasive cellular properties *in vitro* and *in vivo*. Unexpectedly, overexpression of heparanase inhibited brain tumor formation *in vivo* possibly by extensive remodeling of the extracellular matrix which in turn modifies growth factor signaling and activity.

Finally, cell-surface heparan sulfate is also known to inhibit or promote tumorigenesis depending on size and composition. We proposed that heparanase generates bioactive heparan sulfate chains from the melanoma cell-surface that modify biological activities associated with vascular endothelial growth factor, a molecule essential for brain metastasis. Heparanase-degraded melanoma cell-surface heparan sulfate stimulated migration, but not proliferation of melanoma *in vitro.* It also enhanced angiogenesis *in vivo*, independent of vascular endothelial growth factor activity, an unexpected finding. Interestingly, melanoma cell-surface heparan

sulfate did not have an observed effect on endothelioma migration *in vitro*. We also attempted to characterize the melanoma cell-surface heparan sulfate isolated by heparanase degradation by ion-pair high pressure liquid chromatography. This method proved to be not sensitive enough to detect nanogram quantities of HSGAG present in our samples.

CHAPTER I
INTRODUCTION

STATEMENT OF PROBLEM AND HYPOTHESIS

Cancer is the second leading cause of death in the United States, and is a major health concern in the population worldwide [Cancer Facts and Figures, American Cancer Society (2006)]. Cancer metastasis is the spread of tumor cells from a primary site of origin to distant organs and it accounts for 90% of cancer related mortalities. The brain is a frequent site of metastasis from various primary tumors including malignant melanoma (Graf, Buchberger et al. 1988). Malignant melanoma has had the highest increase in incidence in the US over the last three decades, especially among young adults, and is the primary cause of skin cancer related mortalities owing to metastasis (Bogenrieder and Herlyn 2003).

Although metastatic mechanisms are not completely identified, it has been demonstrated that metastasis is not a random event. In stead, it is the result of a complex series of events that occurs between normal cells, tissues, endothelial cells and malignant cells (Nakajima, Irimura et al. 1988; Bogenrieder and Herlyn 2003). In order to metastasize, cells must first detach from the primary tumor, enter the vasculature, circulate through the blood, attach to a vessel wall, exit the vasculature, and establish themselves in new surroundings producing primary metastasis. This whole process may then be repeated to give rise to secondary metastasis (Nicolson 1982; Fidler 2003). The processes of intravasation or extravasation requires degradation of basement membranes, extracellular matrices (ECM), and connective tissue mediated by proteolytic enzymes such as heparanase (HPSE), matrix metalloproteases (MMPs), and/or aspartic, cysteine, and serine proteases (Nakajima, Irimura et al. 1988; Joyce, Freeman et al. 2005).

Heparan sulfate proteoglycans (HSPG) are one of the main components of the cell-surface and ECM. These are macromolecules composed of a core protein with heparan sulfate

chains (HS) covalently attached to it. HSPG are ubiquitous in the eukaryotic system. Substantial evidence accumulated over the last three decades indicates that HSPG act to inhibit invasion by promoting tight cell-cell and cell-ECM interactions (Bernfield, Gotte et al. 1999; Iozzo 2001; Sanderson 2001). Degradation of HS chains weakens cell-cell and cell-matrix adhesion in melanoma and other tumor cells (Dhodapkar, Kelly et al. 1997; Ma and Geng 2000; Engbring, Hoffman et al. 2002). HSPG also act both as reservoirs of growth factors and as co-receptors for ligand binding and subsequent intracellular signaling (Iozzo, Cohen et al. 1994; Vlodavsky and Friedmann 2001). These heparin-binding factors are involved in growth, invasion, angiogenesis, and tumor progression ((Bernfield, Gotte et al. 1999; Iozzo 2001; Vlodavsky and Friedmann 2001).

HS degradative enzymes, such as heparitinases, heparinases from *Flavobacterium heparinum*, or endoglucosaminidases, cleave HS to di- and tetra-saccharides, which are too short for growth factor receptor and ECM ligand binding (Ernst, Langer et al. 1995; Ernst, Venkataraman et al. 1996; Liu, Shriver et al. 2002). On the other hand, HPSE is an endo-β-D-glucuronidase that cleaves HS at specific intrachain sites resulting in fragments of appreciable size (10-20 sugar units) (Nakajima, Irimura et al. 1984; Nakajima, Irimura et al. 1988; Marchetti 1997; Vlodavsky and Friedmann 2001). HPSE has been recently cloned as the only mammalian HS-degrading enzyme (Hulett, Freeman et al. 1999; Kussie, Hulmes et al. 1999; Toyoshima and Nakajima 1999; Vlodavsky, Friedmann et al. 1999). HPSE degrades HS chains of HSPG on the cell surface and ECM of a wide range of normal and neoplastic tissues (Rapraeger 1995; Iozzo 1998; Bernfield, Gotte et al. 1999; Iozzo 2001; Sanderson 2001). Degradation of HSPG by HPSE weakens the cell-matrix barrier and releases growth factors and cytokines bound to HS which can then initiate signaling events necessary for tumor progression (Sanderson, Yang et al.

2004; Sanderson 2005). Increased HPSE expression has been associated with increased growth, metastasis, and angiogenesis of tumors (Vlodavsky and Friedmann 2001). This suggests the notion that by remodeling HSPG, HPSE releases HS-bound angiogenic/growth factors and modulates growth factor activities in metastasis (Marchetti, Denkins et al. 2003; Reiland, Sanderson et al. 2004). We proposed to test the hypotheses 1) whether altering HPSE protein expression will alter metastatic phenotype of melanoma and 2) whether HPSE supports tumorigenesis by releasing bio-active HS from the melanoma cell-surface.

STATEMENT OF RESEARCH OBJECTIVES

The overall goal of this project was to investigate the importance of HPSE and HPSE-degraded HS chains as critical regulators of melanoma tumorigenesis. The specific aims of this research were as follows:

I. Modulate *hpse* gene expression in melanoma by an adenoviral gene delivery method to upregulate or downregulate its expression and determine the following biological effects *in vitro* and *in vivo*:

   1. Establish if *hpse* gene expression could be increased or decreased by adenoviral vectors carrying the gene in sense and anti-sense orientations, respectively and determine if HPSE protein expression and activity is modified accordingly.
   2. Determine if altered HPSE expression enhances or inhibits invasion and metastasis *in vitro* and *in vivo*, respectively.

II. Determine if HPSE-degraded melanoma cell-surface HS act as promoters of vascular endothelial growth factor (VEGF)-mediated activity in melanoma:

   1. Determine if HPSE-degraded cell surface HS modifies VEGF-mediated melanoma migration and proliferation.

2. Determine if HPSE-degraded cell surface HS modifies VEGF-mediated angiogenesis *in vivo*.
3. Characterize the HPSE-degraded cell surface HS products.

On the whole, the results obtained from this research indicate the following: HPSE expression and activity can be modified to alter the metastatic phenotype of melanoma; and, HPSE-degraded melanoma cell-surface HS are bio-active and augment cell migration and angiogenesis independent of VEGF in melanoma. The research is presented in a manuscript format in individual chapters with an appropriate title for the fundamental theme of each chapter.

Chapter II: Antisense-Mediated Suppression of Heparanase Gene Inhibits Melanoma Cell Invasion

Chapter III: Overexpression of Heparanase Inhibits Brain-Metastasis *In Vivo*

Chapter IV: Heparanase-Degraded Cell-Surface Heparan Sulfate Promotes Melanoma Cell Migration and Angiogenesis

LITERATURE REVIEW

Cancer Metastasis: The "Seed and Soil" Hypothesis

Cancer metastasis accounts for 90% of cancer-related deaths and is the second leading cause of death in the United States. Understanding the metastatic process at the systemic, cellular, and molecular levels is essential in cancer research (Bogenrieder and Herlyn 2003; Marchetti, Denkins et al. 2003). Researchers have been studying the process of cancer metastasis for more than 100 years now, and are slowly beginning to understand the mechanisms by which metastatic cells arise from the primary tumors or the site of origin, as well as why certain tumor types have a propensity to metastasize to specific organs.

In 1889, an English surgeon, Stephen Paget, proposed that metastasis depends on cross-talk between selected cancer cells that he termed as the 'seed's and specific organ microenvironments that he termed as the 'soil'. His idea that metastasis is a non-random process still holds forth today (Paget 1889). Paget noticed the discrepancy in the relative blood supply and the frequency of metastases in selected organs. He analyzed more than 900 autopsy records of patients with various primary tumors. His study indicated a non-random pattern of metastasis to visceral organs and bones. To Paget, these findings suggested that the occurrences of metastasis were not due to chance, as was the popular perspective of that time, but that certain tumor cells or the 'seed's have distinct affinity for certain host organs or the 'soil'. His conclusions were that the seed and soil had to be compatible in order to give rise to metastases (Paget 1889).

In 1929, James Ewing confronted Paget's 'seed and soil' theory, and suggested that metastases result simply due to mechanical factors such as the anatomical configuration of the vascular system (Ewing 1928). This theory was accepted till the 1970s, when the selective nature of metastasis was documented by Hart and Fidler (Hart and Fidler 1980; Hart, Talmadge et al. 1981). Analysis of experimental metastasis in syngeneic mice established that mechanical arrest of tumor cells in the capillary bed of remote organs was definitely important, but subsequent proliferation and growth leading to secondary lesions were indeed influenced by the specific organ environment (Hart and Fidler 1980). Further clinical data analyses on site preferences of metastases from different human malignancies suggested that regional metastatic involvement could be explained by anatomical or mechanical considerations. For example, efferent venous circulation or lymphatic drainage leads to metastasis formation in regional lymph

nodes, but metastases to distant organs from various types of primary tumors were indeed site specific (Sugarbaker 1979; Weiss 2000).

In 1989, during a symposium honoring the centennial anniversary of Paget's 'seed and soil' hypothesis, George Poste commented, "There are few scientists, historical or contemporary, whose work will withstand 100 years of scrutiny and not succumb to the depressing trend of modern publications — to ignore papers published more than five years ago". Finally, both Paget's and Ewing's hypotheses proved to be correct and overlapping to some extent to this date. In essence, in order for a tumor cell to metastasize, its interactions with the host homeostatic factors are essential to promote tumor cell growth, survival, angiogenesis, invasion and metastasis.

Cancer metastasis is a well-organized process (Fig. 1.1) (Nicolson 1982; Fidler 2003; Marchetti, Denkins et al. 2003); namely, benign neoplasms undergo a series of sequential but distinct changes to reach a malignant state. For example, a tumor can initially be benign and then become malignant through acquisition of mutations before proceeding all the way through the metastatic cascade. The mutations acquired lead to abnormal and uncontrolled proliferation. Once the tumor reaches the size of 1-2 mm, it needs vascular support (angiogenesis), to maintain tumor viability (Folkman 1986). In order to metastasize, cells must first detach from the primary tumor, enter the vasculature or intravasate, circulate through the blood, attach to vessel walls, exit the vasculature or extravasate, and establish in a new surrounding, producing primary metastasis. This whole process is then repeated to give rise to secondary metastasis (Fig. 1.1) (Nicolson 1982; Fidler 2003). The steps of angiogenesis, arrest and adhesion, and intravasation or extravasation involve subtle to extensive cell-surface degradation and remodeling of the ECM by various proteolytic enzymes secreted by the tumor cells (Liotta 1986).

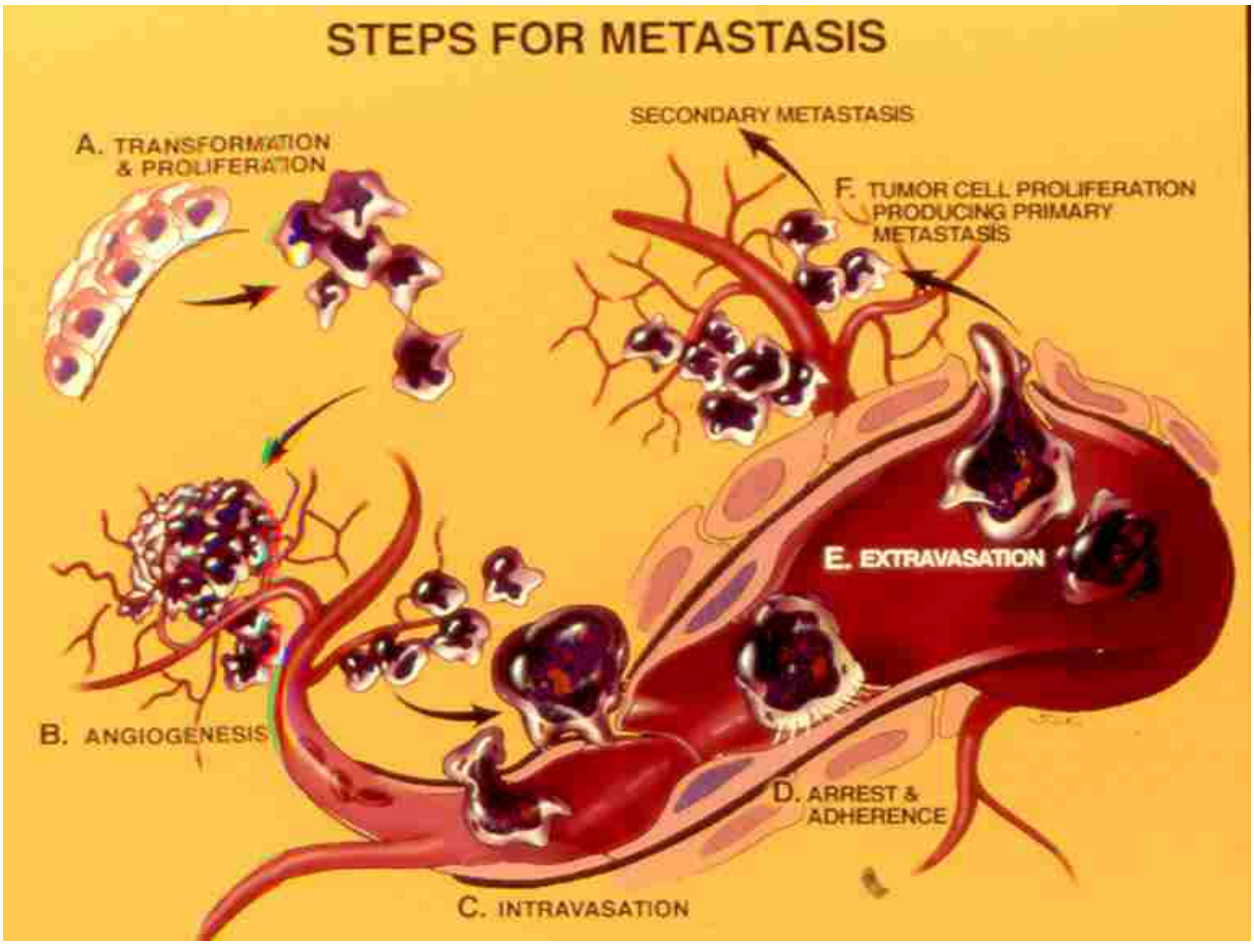


Figure 1.1. The steps of metastasis. A. Cellular transformation and tumor growth. Growth of neoplastic cells for expanding tumor mass is initially supplied by simple diffusion from the surrounding tissue. B. Angiogenesis must occur once a tumor mass dimension exceeds 1–2 mm. in diameter. The synthesis and secretion of angiogenic factors launch a capillary network from the surrounding host tissue. C. Local invasion of the host stroma by some tumor cells that have detached themselves from the primary tumor by several parallel mechanisms to enter the circulation. Thin-walled venules and lymphatic channels that proffer very little resistance to penetration by tumor cells are often the most common route for intravasation. D. On entering the circulation, most tumor cells die. A few surviving tumor cells travel through the vasculature and subsequently become trapped in the capillary beds of distant organs by sticking either to capillary endothelial cells or to subendothelial basement membrane. E. Extravasation occurs consequently by mechanisms similar to those of invasion. F. Adherence and proliferation in the distant organ parenchyma completes the primary metastatic process. The steps are then repeated by the tumor cells to produce additional/secondary metastases [reproduced with permission from Marchetti, D., Denkins, Y. et al. (2003), "Brain-metastatic melanoma: a neurotrophic perspective." Pathol Oncol Res 9(3): 147-58].

Metastatic Melanoma

Malignant melanoma has the highest increase in incidence over the past three decades in the US population, and is the primary cause of skin cancer-related mortalities due to metastasis [Cancer Facts and Figures, American Cancer Society (2006)]. Based on clinical and histopathological features, melanoma progression occurs in five steps (Fig. 1.2) (Clark, Elder et al. 1984; Meier, Satyamoorthy et al. 1998): 1) common acquired and congenital nevi with structurally normal melanocytes, 2) dysplastic nevus with structural and architectural atypia, 3) early radial growth phase (RGP) primary melanoma, 4) advanced vertical growth phase (VGP) primary melanoma with competence for metastasis, and 5) metastatic melanoma. Progression from a resting melanocyte to a common acquired nevus occurs frequently and does not involve genetic abnormality. However, it is proposed that melanocytes progress to a nevus by escaping the keratinocyte surveillance (Valyi-Nagy, Hirka et al. 1993). Keratinocytes control growth, morphology, and antigenic phenotype of melanocytes by establishing direct contact through the cell-cell adhesion receptor E-cadherin (Valyi-Nagy, Hirka et al. 1993). E-cadherin expression is reduced early in the nevus stage and is lost in the majority of melanomas whereas N-cadherin is upregulated in nevi and melanomas; this shift in cadherin profile changes cellular adhesive properties and promotes communication with N-cadherin-expressing fibroblasts and endothelial cells while keratinocytes lose control (Hsu, Wheelock et al. 1996; Meier, Satyamoorthy et al. 1998).

Progression from dysplasia to RGP primary melanoma happens spontaneously without additional molecular changes; however, switch from RGP to metastasis competent VGP is biologically and clinically complicated (Satyamoorthy, DeJesus et al. 1997; Meier, Satyamoorthy et al. 1998). Additionally, VGP cells have growth characteristics comparable to

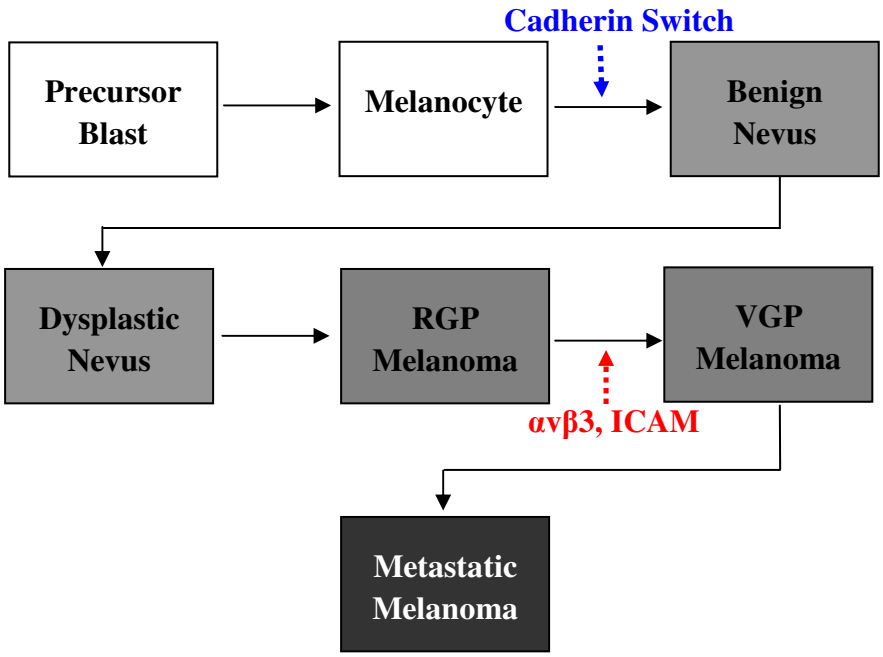


Figure 1.2. Maturation, differentiation, transformation and progression of melanocytic tumors. The role of melanoblasts in melanogenesis remains unknown. Melanoma develops and progresses stepwise from nevic lesions; progression from normal melanocyte to nevus could be initiated by loss of keratinocyte control or contact between melanocytes and keratinocytes due to loss of E-cadherin expression and upregulation in N-cadherin expression (blue arrow). Genetic changes that allow transition from commonly acquired (benign) nevus to dysplastic nevus/RGP melanoma are yet to be identified. Additional genetic changes promote the progression from RGP melanoma to VGP along with expression of adhesion molecules like αvβ3, ICAM (red arrow). At the VGP (tumorigenic) step, increased growth, invasion and stromal proteolysis finally lead to metastasis (adapted from Clark, Elder et al. 1984; Meier, Satyamoorthy et al. 1998).

metastatic cells including anchorage-independent growth in soft agar and tumor formation in immunodeficient mice (Kath, Jambrosic et al. 1991). VGP primary melanomas also exhibit cytogenetic abnormalities, suggesting genomic instability, and they can achieve metastatic phenotype through selection in growth factor-free medium or by induction of invasion *in vitro* through artificial basement membranes. This suggests that alterations in cell–matrix and cell–cell signaling are critical for progression to the metastatic phenotype (Fig. 1.2) (Kath, Jambrosic et al. 1991; Meier, Satyamoorthy et al. 1998).

About 40%-60% of melanoma cases fatally metastasize to the brain (Isaiah, Gabriele et al. 1999; Bogenrieder and Herlyn 2003). The brain is a unique organ confined within the skull and is surrounded by the blood brain barrier (BBB). The BBB acts as a selective diffusion barrier at the level of the cerebral microvascular endothelium and is characterized by the presence of tight cell-cell junctions and a lack of fenestrations in the capillaries (Fenstermacher, Gross et al. 1988; Uwe and Hartwig 2000). Therefore, the brain metastatic tumor cells first have to breach the BBB in order to invade into the brain tissue. Metastases to most parts of the body are silent and hence generally well-tolerated and unnoticed initially, but due to its confined nature, brain tumor formation is usually symptomatic and results in rapid deterioration in health (Menter, Herrmann et al. 1994). The symptoms are characteristically due to cerebral edema which is further aggravated by the lack of a lymphatic circulation in the brain. The resulting deregulation of homeostasis leads to impaired normal brain function and warrants treatment which is often difficult but necessary (Sawaya, Ligon et al. 1996; Soffietti, Ruda et al. 2002).

The rising incidence of melanoma and the high rate of brain metastasis alone would be adequate grounds for alarm, but together they make the area of brain-metastatic melanoma a particularly challenging biological and clinical problem (Nicolson, Menter et al. 1996).

Therefore, melanoma is one of the best studied solid tumors with respect to tumor invasion and metastatic spread in humans (Hofmann, Houben et al. 2005). In view of the fact that the mechanisms for melanoma metastasis to the brain are not yet fully understood, it is extremely important to gain better insights into the molecular mechanisms responsible in order to devise successful treatment strategies. The various steps of metastasis involve cell-surface remodeling and degradation of the ECM by proteolytic enzymes, an important tumor-host interaction in the metastatic cascade (Liotta 1986). The ECM, HSPG, and HPSE are critical in this aspect of tumorigenesis (Bernfield, Gotte et al. 1999; Iozzo 2001; Sanderson 2001; Vlodavsky and Friedmann 2001; Sasisekharan, Shriver et al. 2002; Sanderson, Yang et al. 2004; Sanderson 2005).

Extracellular Matrix

The extracellular matrix (ECM) is a fibrillar meshwork of a variety of proteins and polysaccharides secreted by the cells. The major components of the ECM are the structural proteins collagens and elastin; cell adhesive or anti-adhesive proteins such as laminin, fibronectin, vitronectin, and tenascin; the proteoglycans that have a complex protein core with even more diverse glycosaminoglycan (GAG) side-chains attached to them; and the soluble signaling molecules such as growth factors, cytokines, and chemokines that are bound to the ECM by the heparan sulfate glycosaminoglycans (HSGAG) (Lin and Bissell 1993; Lin and Perrimon 2000). The ECM not only provides a physical barrier between cells and tissues and supports tissues with mechanical strength and elastic properties, it also acts as a scaffold for different cellular functions including cell growth, migration, differentiation and survival (Taipale and Keski-Oja 1997). Development of a multicellular organism from a single cell involves an enormous amount of complexity and cell-cell and cell-ECM communication between different

cells, and organ systems. The ECM provides a structural basis for multicellularity, whereas soluble and insoluble hormones and growth factors transfer the information required for construction of complex cellular structures (Taipale and Keski-Oja 1997). Interaction of ECM molecules with each other or with their specific receptors on the cell surface activated by ligand binding leads to rearrangement of the cytoskeletal network. This, in turn, stimulates signal transduction cascades leading to alterations in gene expression and subsequent biological activity (Lin and Perrimon 2000; Xinhua and Norbert 2002).

Synthesis of ECM is highly regulated by the cells whereby modification and processing of signals occurs at the cell surface leading to altered cell function in response to stimuli (Sasisekharan and Venkataraman 2000). The ECM is remodeled during development and under pathological conditions such as wound healing and cancer (Timpl and Brown 1996). ECM-remodeling enzymes are therefore likely to affect cell and tissue function and are tightly regulated by the cells under normal physiological conditions. However, in a cancerous state, these enzymes are deregulated (Liotta 1986, Stetler-Stevenson, Aznavoorian et al. 1993; Werb 1997). While intensive research has been done on enzymes capable of degrading and remodeling the protein components in the ECM (e.g. matrix metalloproteases, aspartic, cysteine, and serine proteases) (Stetler-Stevenson, Aznavoorian et al. 1993; Werb 1997), limited attention was paid to glycosidase HPSE that modifies the HSGAG on the HSPG where the melanoma metastasis occur (Sanderson, Yang et al. 2004; Sanderson 2005).

Heparan Sulfate Proteoglycans: Structure and Function

The proteoglycan component of the ECM has received much attention in the development of cancer metastasis, as it is present both in the ECM and at the cell surface, and is directly implicated in the cell-tissue interface (Sanderson, Yang et al. 2004; Sanderson 2005).

Proteoglycans play important roles in both how the cell processes signals that arise from the cell-surface and the ECM, and how a signaling molecule once released from the cell-surface, diffuses to a distant site and is processed there (Liu, Shriver et al. 2002). Proteoglycans are a superfamily of glycoproteins that are distinguished by the covalent attachment of one or more GAG chains to the protein core. Four classes of GAGs have been identified so far on the proteoglycans: heparan sulfate (HS)/heparin, chondroitin sulfate (CS), dermatan sulfate (DS), and keratan sulfate (KS) (Kjellen and Lindahl 1991). The proteoglycans of interest are usually HSPG since all eukaryotic cells express HSPG at their cell surface. Genetic mutants that disrupt HSPG core protein synthesis and modification demonstrated that HSPG core protein regulates the interaction of cells with extracellular ligands during development (Kramer and Yost 2003).

Two families of polypeptides are known to carry the majority of the cell surface HS on mammalian cells, the syndecans and the glypicans, while perlecan resides in the ECM (Fig. 1.3) (Blackhall, Merry et al. 2001). The four members of the syndecan family (syndecans 1-4) are transmembrane proteins expressed on nearly all cell types through development and adulthood. Syndecan expression can be deregulated under certain pathophysiological conditions, including the processes of tumor onset, progression and metastasis (Sanderson 2001). The six members of the glypican family (glypicans 1-6) are cell-surface HSPG attached to the plasma membrane via glycosylphosphatidylinositol (GPI) anchors. Glypicans are localized in the rafts and caveolae, and may be involved in caveolar uptake of different biomolecules and viruses resulting in selective regulation of cytokine and growth factor signaling (Fransson, Belting et al. 2004). Perlecan, the major HSPG in the ECM, are important in cell adhesion, proliferation, differentiation, glomerular filtration, and during development through modification of growth factor binding and signaling (Jiang and Couchman 2003; Hacker, Nybakken et al. 2005).

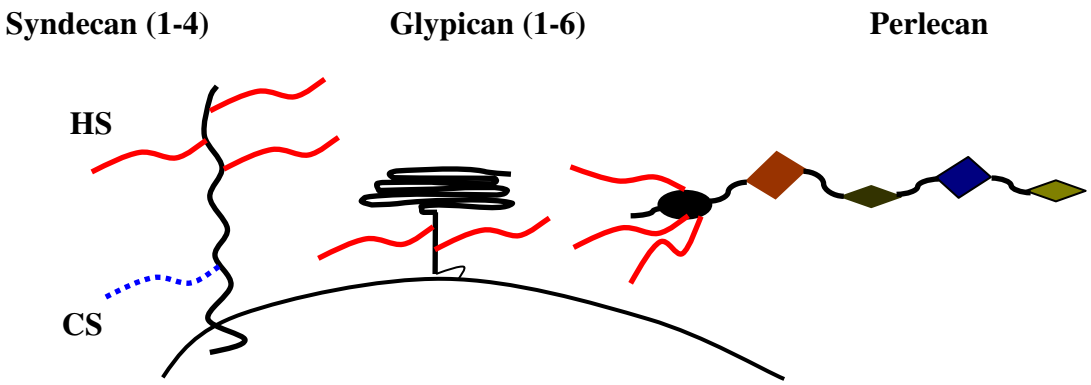


Figure 1.3. Mammalian HSPG. Syndecan 1–4 are encoded by separate genes. These are transmembrane proteins with short cytoplasmic tails with 3-5 HS (Red) are attached near the N-terminus in the ecotodomain. Syndecan 1, 2 and 4 also bear CS (Blue) side chains near the cell surface. The syndecan core proteins are least conserved in the ectodomains even though the HS attachment sites are highly homologous. Six glypican core proteins (Gpc1–6) are encoded by 6 different genes, their extracellular globular glycoprotein cores attach to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor with 2-3 HS chains positioned close to the cell surface. The ECM-resident perlecan consists of a large multidomain core protein (400 kDa) to which several HS chains attach near the terminal globule (adapted from Blackhall, Merry et al. 2001).

During tumorigenesis, these ECM and cell-surface HSPG are remodeled along with the rest of the ECM by various matrix-degrading enzymes including endoglycosidases, matrix metalloproteases, aspartic, cysteine, and serine proteases to facilitate tumor cell proliferation, angiogenesis, invasion, and metastasis (Joyce, Freeman et al. 2005). HSPG promote tumor progression by releasing their HS-bound growth factors and cyto/chemokines that are now capable to signal through their cognate receptors, combined with alterations in the BM/ECM, that facilitate angiogenesis and tumor invasion by enzymatic action of HPSE (Fig. 1.4) (Sanderson 2001; Sanderson, Yang et al. 2004; Joyce, Freeman et al. 2005; Sanderson 2005).

Heparan Sulfate Glycosaminoglycans: Structure and Function

HSGAG are ubiquitously expressed on the surface of all eukaryotic cells and are important regulators of normal and pathological physiology (Sasisekharan, Shriver et al. 2002). HSGAG are synthesized in the Golgi apparatus starting with four monosaccharides linked to a serine-glycine repeats flanked by hydrophobic and acidic domains on the core protein. The typical linear polysaccharide is then synthesized by the sequential addition of a glucosamine linked to an uronic acid with many possible disaccharide unit combinations (Gallagher 2001). These different combinations are based on the fact that *O*-sulfation can occur at the 2-*O* position of uronic acid and at the 6-*O* and 3-*O* positions of the glucosamine. This creates eight different arrangements because each site can be either sulfated or unsubstituted plus the possibility for sixteen additional different combinations of disaccharides resulting from the uronic acid component being either iduronic acid or glucuronic acid. Finally, the *N*-position of the glucosamine can either be sulfated, acetylated, or unsubstituted, resulting in 48 varied possibilities for disaccharide structures (Fig. 1.5) (Esko and Lindahl 2001).

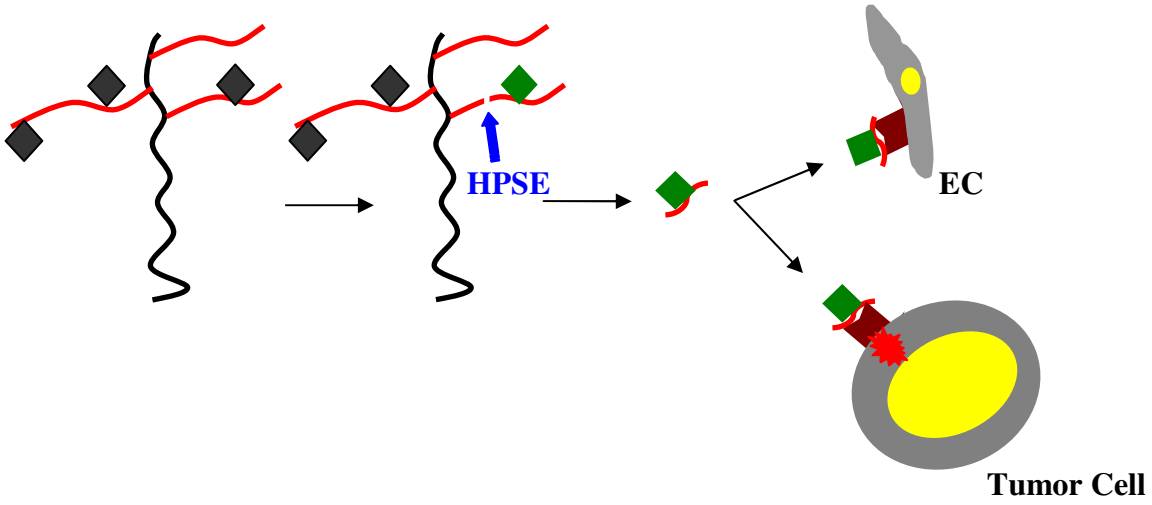


Figure 1.4. Model of endoglycosidase HPSE function within the tumor microenvironment. HPSE cleaves HS chains (Red) releasing growth factors bound to HS fragments. HPSE activity on HS augments the biological activity of growth factors with resulting stimulation of cell proliferation (Kato, Wang et al. 1998; Liu, Shriver et al. 2002). Growth factors released by HPSE can act on both tumor cells and endothelial cells (EC) (adapted from Sanderson, Yang et al. 2004).

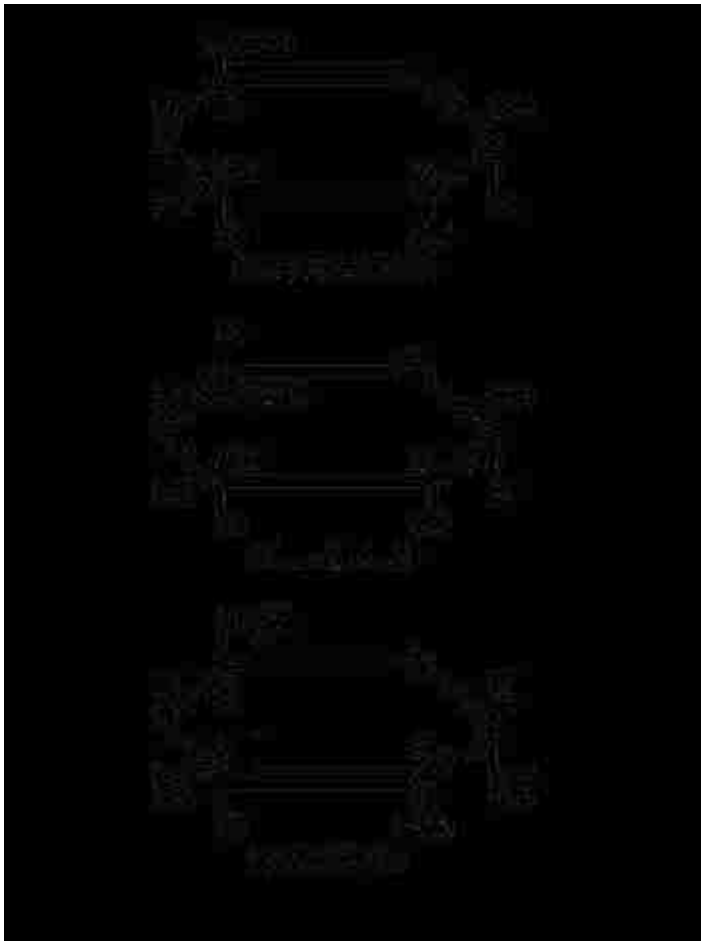


Figure 1.5. Disaccharide sequences present in heparan sulfate. A glucosamine is alternately linked to a uronic acid (either glucuronic acid or iduronic acid) with many possible disaccharide unit combinations (Gallagher 2001). HS predominantly contain glucuronic acid over iduronic acid. The different combinations are based on the fact that *O*-sulfation can occur at the 2-*O* position of uronic acid and at the 6-*O* and 3-*O* positions of the glucosamine. This creates eight different arrangements because each site can be either sulfated or unsubstituted leading to the prospect for sixteen additional different combinations of disaccharides by the fact that there are two possibilities for the uronic acid component which can be either iduronic acid or glucuronic acid. Finally, the *N*-position of the glucosamine can be sulfated, acetylated, or unsubstituted, resulting in 48 varied possibilities for disaccharide structures (R = –H or $–SO_3$, X = -Ac or $–SO_3$, Y = –OH or $–OSO_3$, Z = –OH or $–OSO_3$).

The immense structural diversity of HSGAG allows them to bind and interact with numerous proteins including growth factors, chemokines, morphogens, and enzymes. Growth factors including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and, insulin-like growth factor are known to be important for tumor development (Nurcombe, Smart et al. 2000; Iozzo 2001; Sasaki, Higashi et al. 2004). The specificity of growth factor binding and interactions is dependent on the HSGAG sequence including sulfation pattern, spacing of binding sites, and the three-dimensional structure of the HSGAG chain (Rapraeger 1995; Sasisekharan, Shriver et al. 2002; Robinson, Mulloy et al. 2006). HSGAG present on tumor cells also contain bioactive sequences that may affect tumor-cell phenotype in relation to cell growth and metastasis (Liu, Shriver et al. 2002; Sasisekharan, Shriver et al. 2002). The expression pattern and the structural integrity of HSGAG on the cell surface may change with the metastatic transformation of certain cell types; e.g., B16 melanoma cells with high metastatic propensities have a lower concentration of cell surface HSGAG than sublines with less metastatic potential (Kure, Yoshie et al. 1987). Highly metastatic B16 sublines also degrade HSGAG faster than sublines of lower metastatic spread (Nakajima, Irimura et al. 1983).

HSGAG 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 normal host cells, HSGAG mediate tumor and host cell interactions (Raman, Venkataraman et al. 2003). HSGAG can also influence tumor metastasis to sites such as the brain by mediating interactions between cancer cells, platelets, endothelial cells, and host organ cells (Varki and Varki 2002). When intact, HSGAG prevent metastasis by acting as a physical barrier in the ECM. HPSE secreted by tumor cells degrades HSGAG on the cell-surface and ECM to increase the

invasiveness of tumor cells both by breaching the physical barrier as well as by releasing the HS-bound growth factors that aid in the metastatic process (Vlodavsky and Friedmann 2001; Sanderson 2005).

Heparanase in Health and Disease

Discovery

Heparanase or HPSE is an endo-β-D-glucuronidase that cleaves HS side chains at a limited number of intra-chain sites yielding HS fragments of 5–7 kDa (Fig. 1.6) (Freeman and Parish 1998; Pikas, Li et al. 1998; Vlodavsky and Goldshmidt 2001). HPSE activity was first identified in rat liver tissue (Hook, Wasteson et al. 1975). Later it was found that HPSE activity correlated with increased lung metastatic potential in B16 sublines (Nakajima, Irimura et al. 1983), and in aggressive lymphoma (Vlodavsky, Fuks et al. 1983). Since then, HPSE activity has been linked to a wide number of primary and metastatic cancers including cancers of the bladder, colon, stomach, breast, pancreas, brain, and melanoma (Friedmann, Vlodavsky et al. 2000; Gohji, Okamoto et al. 2001; Koliopanos, Friess et al. 2001; Marchetti and Nicolson 2001; Maxhimer, Quiros et al. 2002; Tang, Nakamura et al. 2002; Simizu, Ishida et al. 2004). HPSE activity correlates with the metastatic potential of all tumor-derived cells studied so far, possibly due to enhanced cell dissemination resulting from HS cleavage and remodeling of the cell-surface and the ECM (Parish, Freeman et al. 2001; Vlodavsky and Friedmann 2001). Notably, HPSE activity has also been detected in platelets, endothelium, macrophages, astrocytes and placenta which implicates its role in normal physiological functions such as neovascularization, inflammation, autoimmunity, and embryonic implantation (Matzner, Vlodavsky et al. 1992; Marchetti, Li et al. 2000, Dempsey, Brunn et al. 2000; Parish, Freeman et al. 2001; Vlodavsky and Friedmann 2001; Revel, Helman et al. 2005). The enzyme is preferentially expressed in the

Figure 1.6: Glycosaminoglycan sequence recognized by endoglycosidase HPSE. The exact order of iduronic acid, glucosamine and glucuronic acid residues recognized by HPSE is yet to be determined. Heparan sulfate with unsubstituted glucosamine residues is not cleaved by HPSE. On the other hand, heparan sulfate with glucosamines modified by *N*-acetylation or *N*-sulfation is susceptible to cleavage by HPSE. Hexuronic acid carboxyl groups are required for substrate cleavage but are dispensable for recognition because carboxyl-reduced heparan sulfate is a potent inhibitor; *O* sulfation is not an absolute requirement for cleavage, provided that the glucosamine residue is *N*-substituted (Bai, Bame et al. 1997; Freeman and Parish 1998; Pikas, Li et al. 1998). Enzymatic cleavage produces a glucuronic acid at the newly formed reducing terminus (marked by the arrow) [reproduced with permission from Dempsey, Brunn et al. (2000), "Heparanase, a potential regulator of cell-matrix interactions. Trends in Biochemical Sciences *25*, 349-351"].

cells of developing vascular and nervous systems during embryogenesis (Goldshmidt, Zcharia et al. 2001). HPSE also promotes migration of endothelial cells and activated immune cells suggesting that HPSE has important roles in both pathologic and non-pathological conditions (Nakajima, Irimura et al. 1988; Matzner, Vlodavsky et al. 1992; Schubert, Ilan et al. 2004).

HPSE activity was initially attributed to proteins with molecular weights varying from 8 to 130 kDa, therefore raising the potential existence of several HS-degrading endoglycosidases (Dempsey, Brunn et al. 2000; Parish, Freeman et al. 2001; Vlodavsky and Friedmann 2001). However, cloning of a single human *hpse* cDNA sequence by several groups independently (Hulett, Freeman et al. 1999; Kussie, Hulmes et al. 1999; Toyoshima and Nakajima 1999; Vlodavsky, Friedmann et al. 1999) suggested that unlike the family of proteases such as the large family of matrix metalloproteases that can digest different components of the ECM, HPSE (also referred to as HPSE-1) is known to be the only mammalian endoglycosidase. The unique characteristic of this enzyme makes it an attractive target for treatment of various malignancies.

Molecular Properties

The human *hpse* gene (~50 kb) is located on chromosome 4q21.3 and is expressed as 5 and 1.7 kb mRNA species, generated by alternative splicing (Dong, Kukula et al. 2000). The 5 kb mRNA form contains 14 exons separated by 13 introns, whereas in the short 1.7 kb form, the first and 14^{th} exons have been spliced out. Sequence analysis suggested that HPSE is highly conserved across mammalian species with about 80% homology between human, rat, mouse and bovine sequences and about 60% homology with the chicken sequence (Goldshmidt, Zcharia et al. 2001; Parish, Freeman et al. 2001). The human *hpse* cDNA contains an open reading frame encoding a polypeptide of 543 amino acids with a predicted molecular weight of 61.2 kDa. The active HPSE purified from placenta, platelets and various cell lines lacked the N-terminal

domain of 156 amino acids (Toyoshima and Nakajima 1999; Vlodavsky, Friedmann et al. 1999; Goldshmidt, Zcharia et al. 2001; Parish, Freeman et al. 2001) suggesting post-translational modification. Thus, HPSE is synthesized as an inactive precursor which is processed proteolytically to generate an active enzyme (Fairbanks, Mildner et al. 1999). The intervening 6 kDa peptide (Ser110–Gln157) is excised to generate a heterodimer consisting of a 50 kDa subunit (Lys158–Ile543) attached noncovalently to an 8 kDa peptide (Gln36–Glu109) (Fairbanks, Mildner et al. 1999; Levy-Adam, Miao et al. 2003; McKenzie, Young et al. 2003) (Fig. 1.7). Notably, the 8 kDa N-terminal sequence is required for enzymatic activity; when expressed alone, the truncated 50 kDa (Lys158–Ile543) protein fails to demonstrate any enzymatic activity (Hulett, Hornby et al. 2000). Co-transfection and immunoprecipitation studies further indicate that heterodimer formation is essential for enzymatic activity as it is only obtained by co-expression of both the 8 and 50 kDa subunits (Levy-Adam, Miao et al. 2003; McKenzie, Young et al. 2003). Using site-directed mutagenesis techniques to determine the amino acids critical for cleavage at Glu109– Ser110 (site 1) and Gln157–Lys158 (site 2), Abboud-Jarrous et al. 2005 reported that mutations generated at site 1 and its flanking regions did not effect HPSE processing or activity. Alternatively, substitution of Tyr156 at site 2 by alanine or glutamine rendered HPSE inactive and the enzyme was also processed incorrectly (Abboud-Jarrous, Rangini-Guetta et al. 2005). This study also pointed out that a tyrosine at position 2 (P2) of the cleavage site (Gln157–Lys158) was absolutely necessary for proper HPSE processing and activation, resembling the cleavage specificity of cathepsin L (Abboud-Jarrous, Rangini-Guetta et al. 2005). Interestingly, incubation of the purified latent 65 kDa HPSE with cathepsin L generated appropriately processed and active HPSE, composed of the 50 and 8 kDa subunits. This observation was repressed in the presence of a specific, cell permeable inhibitor

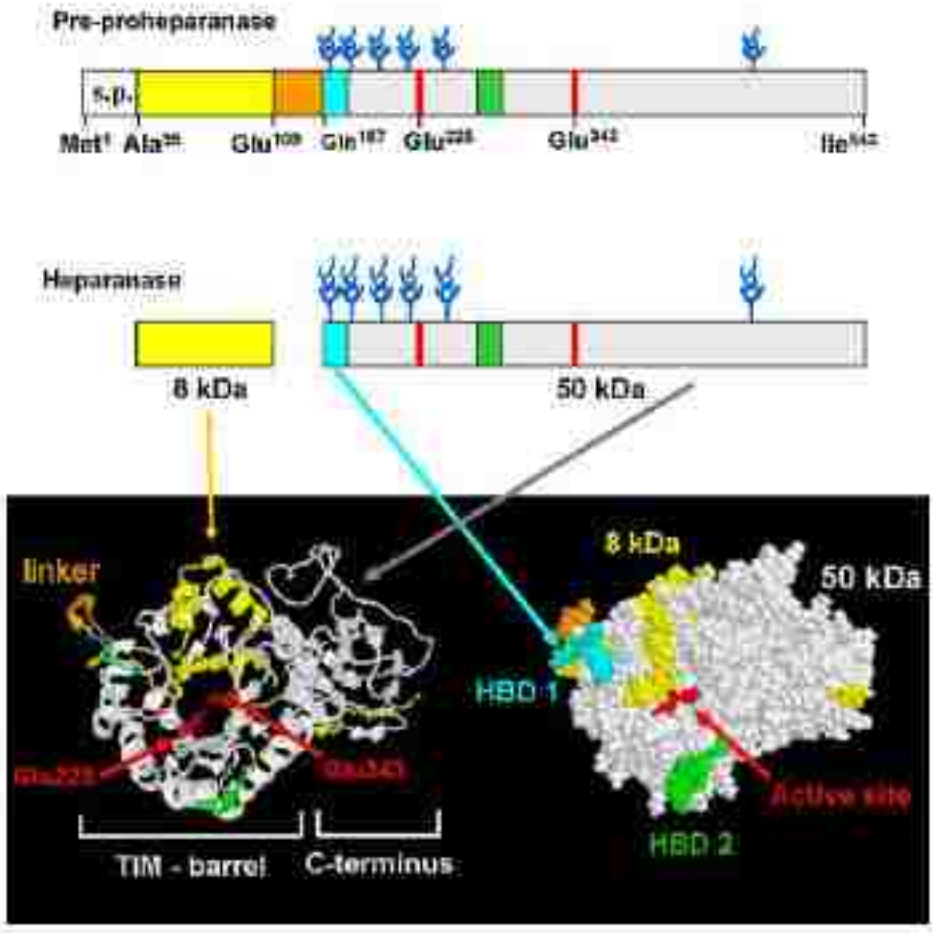


Figure 1.7: Primary structure and predicted 3D structure of the HPSE heterodimer (Ilan, Elkin et al. 2006). N-terminal signal peptide (SP, Met1–Ala35) is removed from pre-proHPSE after entering the ER. The protein is then subjected to glycosylation at six N-glycosylation sites ( ) in the Golgi apparatus followed by secretion as a latent 65 kDa protein (upper panel). Proteolytic removal of the linker domain (Ser110–Gln157) results in an 8 kDa (Gln36–Glu109) and 50 kDa (Lys158–Ile543) protein subunits (middle panel) that heterodimerize to yield the active enzyme. A predicted three-dimensional structure of the HPSE heterodimer (bottom panel) was created based on homology with the endo 1,4-β-xylanase. The 8 (yellow) and 50 kDa (gray) subunits and glutamic acid residues 225 and 343 are illustrated (bottom panel) at the enzyme active site (red). The heparin binding domains (HBD 1 & 2, blue and green) are in close proximity to the enzyme active site (red) (Hulett, Hornby et al. 2000; Nardella, Lahm et al. 2004; Ilan, Elkin et al. 2006) [reproduced with permission from Ilan, N., Elkin, M. et al. (2006), "Regulation, function and clinical significance of heparanase in cancer metastasis and angiogenesis." Int J Biochem Cell Biol 38(12): 2018-39].

of cathepsin L (Abboud-Jarrous, Rangini-Guetta et al. 2005). It is possible that cathepsins other than cathepsin L may activate proHPSE in a cell- and tissue-dependent manner. The predicted amino acid sequence of HPSE also suggested six putative *N*-glycosylation sites on the 50 kDa subunit signifying further possible post-translational modification needed for enzymatic activity. Interestingly, glycosylation did not affect enzymatic activity but it was found to be essential for secretion of HPSE (Simizu, Ishida et al. 2004).

HPSE enzyme activity is also closely regulated by pH, achieving maximal activity under acidic conditions at an optimum range between pH 5.5-6.8 (Mckenzie 2003) that correlates well with the concept that the microenvironment in tumors is usually more acidic than in normal tissues. This acidity of the tumor microenvironment is also found within hypoxic areas of growing tumors (Tannock et al., 1989). Upon activation in this acidic environment, HPSE facilitates the release of several protein modulators that affect cell functions such as migration, adhesion, inflammation, and angiogenesis (Sanderson 2005).

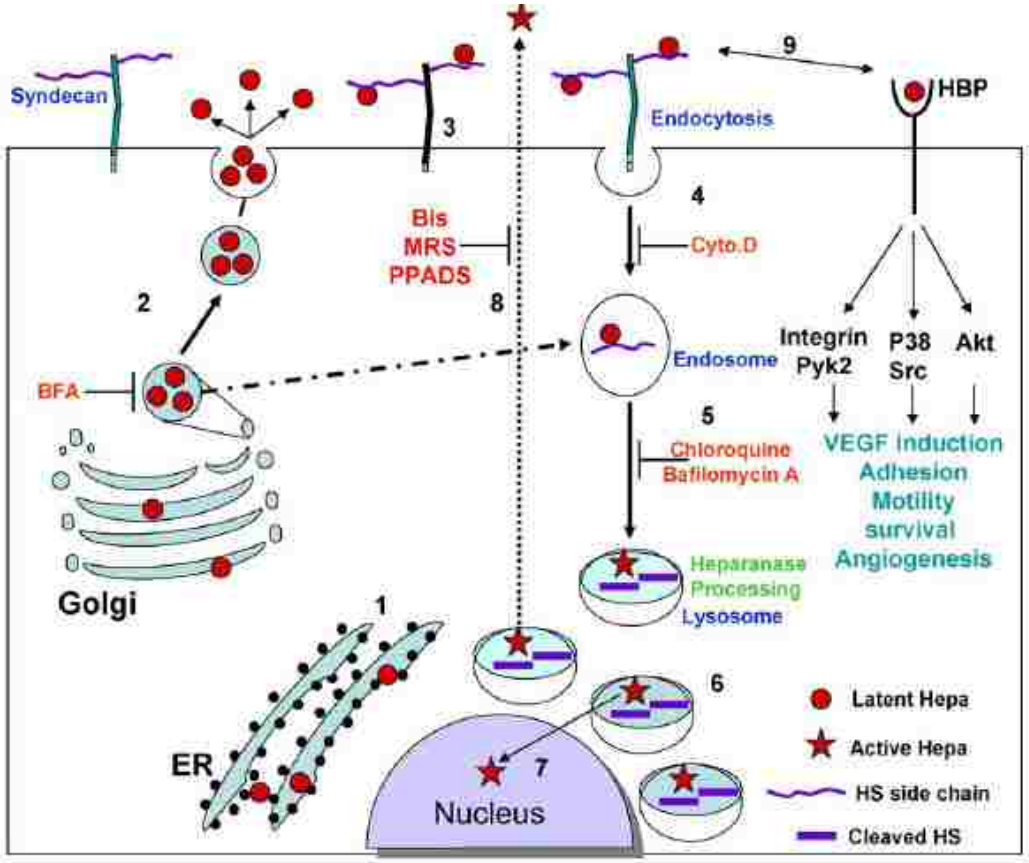
Structure, Processing and Trafficking

According to sequence alignment and secondary structure prediction, HPSE is thought to adopt a TIM (triosephosphate isomerase) barrel fold, similar to other known glycosyl hydrolases (Hulett, Hornby et al. 2000). A TIM barrel fold is usually formed by eight alternating α-helices and β-strands. The 50 kDa HPSE subunit is believed to have six α/β units and, an α/β/α element is predicted to be contributed by the 8 kDa subunit (Nardella, Lahm et al. 2004) (Fig. 1.7). This family of enzymes uses a general acid catalysis mechanism for the hydrolysis of glycosidic bonds that requires two critical residues, a proton donor and a nucleophile, both of which seem to be conserved in HPSE at Glu225 and Glu343, respectively (Hulett, Hornby et al. 2000). Site-directed mutagenesis of these two residues inhibited HPSE activity completely, signifying that

HPSE employs a catalytic mechanism characteristic of GH-A glycosyl hydrolyses (Hulett, Hornby et al. 2000) to cleave the linkage between the glucuronic acid and N-acetylglucosamine of the HSGAG (Nakajima, Irimura et al. 1983).

When added externally to cells in culture, pro-HPSE is rapidly internalized and converted into the active enzyme suggesting that processing may occur at the cell surface (Vlodavsky and Friedmann 2001; Nadav, Eldor et al. 2002), but recent studies indicate that HPSE processing occurs intracellularly in acidic vesicles, most likely lysosomes (Gingis-Velitski, Zetser et al. 2004a; Zetser, Levy-Adam et al. 2004; Vreys, Delande et al. 2005). Following exogenous addition, both active and latent forms of the enzyme localize in endocytic vesicles, suggesting that processing does not occur at the cell surface (Zetser et al., 2004). Despite its localization in the lysosome, HPSE has a half life of about 30 h (Gingis-Velitski, Zetser et al. 2004a) and in response to proper stimuli it may be secreted from the lysosomes (Shafat, Vlodavsky et al. 2006). HPSE uptake is also mediated by cell surface HS in primary fibroblasts and endothelial cells, as well as in tumor cells to restrict its availability on the cell-surface (Nadav, Eldor et al. 2002; Gingis-Velitski, Zetser et al. 2004a; Gingis-Velitski, Zetser et al. 2004b; Zetser, Levy-Adam et al. 2004). When HPSE was overexpressed in HS-deficient cells, latent enzyme accumulated in the culture medium along with decreased levels of the active intracellular processed enzyme suggesting that the latent protein is secreted first followed by HS-mediated endocytosis (Gingis-Velitski, Zetser et al. 2004a) (Fig. 1.8). Sequence alignment indicated two heparin binding domains in HPSE at Lys158–Asp162 at the N-terminus, and at Pro271–Met278 of the 50 kDa subunit (Levy-Adam, Abboud-Jarrous et al. 2005). A peptide containing the Lys158–Asp162 sequence (KKDC) displayed firm binding to heparin and HS and repressed HPSE uptake and activity possibly due to competition with the HS substrate (Levy- Adam, Abboud-Jarrous et al.

Figure 1.8. An anticipated model for HPSE biosynthesis, processing and trafficking (Ilan, Elkin et al. 2006). 1) Pre-proHPSE is targeted to ER by its own signal peptide (Met1–Ala35, 2) The 65 kDa pro-heparanase is then transferred to Golgi apparatus and is subsequently secreted via exocytosis, a step that is specifically inhibited by Brefeldin A (BFA) (Nadav, Eldor et al. 2002). 3) Following secretion, HPSE quickly interacts with cell membrane HSPG such as the syndecans (Gingis-Velitski, Zetser et al. 2004a), mannose-6 phosphate receptor or LRP (Vreys, Delande et al. 2005), 4) followed by a rapid endocytosis of the HPSE-HSPG complex which seems to reside in endosomes; this is inhibited by cytochalasin D (Cyto. D) (Nadav, Eldor et al. 2002), or heparin (Gingis-Velitski, Zetser et al. 2004a). 5) Conversion of endosomes to lysosomes leads to processing and activation of HPSE which, in turn, participate in the turnover of HS in the lysosomes. HPSE processing and activation is specifically inhibited by chloroquine and Bafilomycin A that are inhibitors of lysosomal proteinases (Zetser, Levy-Adam et al. 2004). 6) HPSE at perinuclear lysosomal vesicles arises possibly by direct conversion of secretory vesicles to endosomes (dashed arrow). 7) Lysosomal HPSE may translocate to the nucleus, where it could influence gene transcription, leading to differentiation of carcinoma cells (Takaoka, Naomoto et al. 2003; Ohkawa, Naomoto et al. 2004), or 8) it can be secreted in response to local or systemic cues. Secretion of active HPSE is repressed by PKC inhibitors (Bis), and P2Y receptor antagonists (MRS, PPADS) (Shafat, Vlodavsky et al. 2006). 9) The latent secreted HPSE can also interact with HPSE-binding proteins (HBP) and stimulate signaling components such as Akt, p38, Src, Pyk2 and integrins, leading to enhanced cell adhesion, migration, VEGF induction and angiogenesis (Goldshmidt, Zcharia et al. 2003; Zetser, Bashenko et al. 2003; Gingis-Velitski, Zetser et al. 2004b; Sotnikov, Hershkoviz et al. 2004; Zetser, Bashenko et al. 2006) [reproduced with permission from Ilan, N., Elkin, M. et al. (2006), "Regulation, function and clinical significance of heparanase in cancer metastasis and angiogenesis." Int J Biochem Cell Biol 38(12): 2018-39].


Syndecan
3
9
HBP
Endocytosis
4
Bis
MRS
PPADS
Cyto.D
2
8
Integrin
Pyk2
P38
Src
Akt
BFA
Endosome
5
Chloroquine
Bafilomycin A
VEGF Induction
Adhesion
Motility
survival
Angiogenesis
Heparanase
Processing
Lysosome
Golgi
1
6
Latent Hepa
Active Hepa
ER
7
Nucleus
HS side chain
Cleaved HS

2005). A KKDC deletion mutation resulted in intracellular buildup of the latent form due to impaired secretion, but deletion of the Pro271–Met278 sequence caused accumulation of the pro-enzyme in the medium, suggesting an additional role of HS in HPSE uptake and processing (Levy-Adam, Abboud-Jarrous et al. 2005). The KKDC sequence also seems to dwell in close proximity to HPSE active sites (Glu225 and Glu343) in a micro pocket domain (Fig. 1.7) (Hulett, Hornby et al. 2000). Two additional cell surface receptors, low-density lipoprotein receptor-related protein (LRP) and the mannose 6-phosphate receptor, have also been recognized as mediating HPSE uptake (Vreys, Delande et al. 2005).

Regulation of Gene Expression

Promoter methylation has an important role in HPSE gene transcription. Tumor-derived cell lines exhibiting HPSE activity harbor at least one unmethylated allele (Shteper, Zcharia et al. 2003; Ogishima, Shiina et al. 2005a; Ogishima, Shiina et al. 2005b). Ogishima et al. (2005b) have also demonstrated a correlation between HPSE expression levels in bladder and prostate carcinomas and the expression levels of early growth response 1 (EGR1), a transcription factor important for HPSE gene transcription (de Mestre, Khachigian et al. 2003; de Mestre, Rao et al. 2005). HPSE promoter contains four putative estrogen response elements, and estrogen stimulates HPSE mRNA expression in estrogen receptor-positive human breast carcinoma cells (MCF7), but not in estrogen receptor-negative human breast carcinoma (MDA-MB-231). Estrogen also enhances HPSE-mediated angiogenesis *in vivo* (Elkin, Cohen et al. 2003). CREB (cyclic adenosine monophosphate binding protein), another transcription factor important in tumor progression, regulates HPSE expression and activity in melanoma in a positive manner (Aucoin, Reiland et al. 2004). In addition, the *hpse* gene is constitutively repressed by wild type p53 under normal conditions. This could explain an upregulation of HPSE expression observed

in various primary and metastatic cancers where p53 is often mutated or inactivated in the course of tumor progression (Baraz, Haupt et al. 2006).

Function

HPSE has important normal physiological functions such as neovascularization, inflammation, autoimmunity, and embryonic implantation (Matzner, Vlodavsky et al. 1992; Dempsey, Brunn et al. 2000; Parish, Freeman et al. 2001; Vlodavsky and Friedmann 2001; Revel, Helman et al. 2005). It also has essential pro-angiogenic and pro-metastatic roles attributed to its enzymatic activity. A critical early event during angiogenesis is degradation of the subendothelial basement membrane (BM) which is followed by endothelial cell (EC) migration toward the angiogenic stimulus. HPSE may directly aid EC invasion and sprouting by degrading HS in the BM and by releasing HS-bound angiogenic growth factors (i.e., FGF2, VEGF) from the ECM (Folkman, Klagsbrun et al. 1988) that could indirectly facilitate EC proliferation and migration (Vlodavsky, Miao et al. 1996; Elkin, Ilan et al. 2001). Furthermore, HPSE-degraded bio-active HS fragments can stimulate angiogenesis by modifying activities of angiogenic growth factors (Reiland, Kempf et al. 2006). HPSE activity correlates with the metastatic potential of tumor cells; sub lines with higher metastatic potential exhibit a higher enzymatic activity than low- or non-metastatic cells (Hulett, Freeman et al. 1999; Vlodavsky, Friedmann et al. 1999; Yang, MacLeod et al. 2005). Overexpression of HPSE by transfection or an adenoviral gene delivery method increased invasive and metastatic properties in cancers of esophagus, lung and in melanoma (Uno, Fujiwara et al. 2001; Roy, Reiland et al. 2005). Conversely antisense, siRNA and ribozyme technologies-mediated reduction of HPSE expression led to a decrease in invasive properties *in vitro* and *in vivo* (Uno, Fujiwara et al. 2001; Edovitsky, Elkin et al. 2004; Roy, Reiland et al. 2005). Interestingly, exceedingly elevated

HPSE expression levels can also inhibit tumorigenesis, probably due to extensive cell-surface remodeling (Zetser, Bashenko et al. 2003; Reiland, Kempf et al. 2006).

HPSE also exerts non-enzymatic activities, independent of its involvement in ECM degradation and remodeling. Inactive HPSE expressed on the cell-surface leads to firm cell adhesion, indicating an involvement in cell–ECM interaction (Goldshmidt, Zcharia et al. 2003). Inactive HPSE also enhances Akt signaling and promotes PI3K- and p38-dependent endothelial cell migration and invasion (Gingis-Velitski, Zetser et al. 2004b).

Inhibitors

Low-molecular-weight heparin (LMWH) prolongs survival in patients with cancer according to recently published randomized-controlled trials (RCTs) in which four different types of LMWH improved the survival in patients with advanced cancer (Kakkar, Levine et al. 2004; Klerk, Smorenburg et al. 2005; Rickles 2006). LMWH either directly affects tumor growth and metastasis and/or indirectly inhibits thrombin generation, the latter being widely implicated in tumorigenesis (Rickles, Patierno et al. 2003). Heparin may also inhibit metastasis by blocking platelet–tumor cell interactions, thus inhibiting aggregates of tumor cells being trapped in the microvasculature or by blocking selectin-mediated cell–cell interactions to prevent tumor cells from attaching to the vascular endothelium (Borsig, Wong et al. 2001; Borsig 2004). An array of HPSE inhibiting molecules, including peptides, small molecules, neutralizing antibodies, modified non-anti-coagulant species of heparin, and several polyanionic molecules (laminarin sulfate, suramin analogues and PI-88) have been developed (Marchetti, Reiland et al. 2003; Ferro, Hammond et al. 2004; Simizu, Ishida et al. 2004; Ilan, Elkin et al. 2006). Abnormal expression of HPSE in malignancy, combined with the fact that it is the only mammalian glycosidase involved in tumorigenesis, makes it an attractive target for cancer chemotherapy.

Fibroblast Growth Factor-2 and Vascular Endothelial Growth Factor in Angiogenesis

The term angiogenesis was first introduced by Judah Folkman in 1971 to propose that tumor growth beyond a certain dimension (0.2-2.0 mm or about 10^5-10^6 cells) required formation of new blood vessels from the pre-existing vascular network (Folkman 1971; Folkman, Merler et al. 1971). Angiogenesis contributes to development of the cardiovascular system during embryogenesis, and is essential in adult life for wound healing and the female reproductive cycle. Turnover of endothelial cells is sluggish under normal physiological circumstances; however, tumor endothelial cells divide rapidly and frequently during neoangiogenesis (Folkman 1995; Iozzo and San Antonio 2001). Angiogenesis is a multi-step process that initiates with the localized degradation of the basement membrane by activated endothelial cells that migrate and proliferate. This leads to formation of solid endothelial cell sprouts into the stromal space which grow into vascular loops. Finally capillary tubes are developed with the formation of tight junctions and deposition of new basement membrane (Carmeliet 2000). The transformation of preangiogenic phenotype to the angiogenic phenotype by an "angiogenic switch" results from an imbalance between the pro- and anti-angiogenic factors that are released primarily by surrounding pericytes and lymphocytes (Ranieri and Gasparini 2001). A large number of these factors are known to bind to heparin/HS on HSPG, including key angiogenic growth factors such as the FGFs and VEGFs (Hanahan and Folkman 1996; Iozzo and San Antonio 2001).

FGF1 and FGF2 were the first two prototypic heparin-binding angiogenic growth factors to be identified, purified and sequenced (Folkman, Merler et al. 1971; Maciag, Mehlman et al. 1984; Shing, Folkman et al. 1984). Since then, 22 structurally-related members of the FGF family have been identified (Itoh and Ornitz 2004). FGFs induce cell proliferation and/or differentiation of mesodermal or neuroectodermal origin, including endothelial cells and

melanoma following interaction with heparan-sulfate proteoglycans (HSPG) and tyrosine kinase FGF receptors (FGFRs) (Burgess and Maciag 1989; Gospodarowicz 1991; Rapraeger, Krufka et al. 1991; Dellian, Witwer et al. 1996). *In vivo*, FGF is known to mediate growth and neovascularization of solid tumors (Folkman and Klagsbrun 1987). Antisense FGF1 and FGF2 receptor cDNAs inhibit growth and angiogenesis in human melanoma (Wang and Becker 1997). Cells expressing FGF-2 are known to be more angiogenic than the nonexpressing counterpart (Konerding, Fait et al. 1998). FGF2 is stored in the ECM complexed with the HS (low affinity) on HSPG and this complex formation is required for FGF2 receptor binding (high affinity ternary complex) and subsequent signaling and activity (Reiland and Rapraeger 1993; Rapraeger 1995).

VEGF (vascular endothelial growth factor), also known as vascular permeability factor (VPF) (Senger, Galli et al. 1983; Senger, Perruzzi et al. 1986) was originally described as a highly specific mitogen for endothelial cells (Leung, Cachianes et al. 1989) and as a potent inducing agent for angiogenesis and vasculogenesis in a variety of physiological and pathological conditions (Plate, Breier et al. 1992; Ferrara, Winer et al. 1993; Tuder, Flook et al. 1995; Ferrara and Davis-Smyth 1997). Inhibition of VEGF signaling by VEGF antagonists, or antisense VEGF, or dominant negative VEGFR, impaired tumorigenesis *in vivo* (Kim, Li et al. 1993; Millauer, Shawver et al. 1994; Saleh, Stacker et al. 1996; Yano, Shinohara et al. 2000). The VEGF family of proteins includes VEGF A through E and placenta growth factor (Neufeld, Cohen et al. 1996; Ranieri, Patruno et al. 2006). VEGF A is the most well studied one and one of its splice variants, VEGF165, is the most potent and widely expressed isoform known (Plouet, Moro et al. 1997). VEGF165 is secreted as a disulfide-linked homodimer with two identical heparin-binding sites. Interactions with HS regulate the diffusion, half-life, and affinity of

VEGF165 for its signaling receptors. VEGF165 binding to VEGFR1 is dependent on cellular HSPG; exogenous heparin/HS cannot fully compensate for the loss of cell-surface HS, suggesting that HSPG play a role in VEGF165 presentation (Terman, Khandke et al. 1994; Cohen, Gitay-Goren et al. 1995). VEGF165 can bind VEGFR2 in the absence of HS, but this interaction is enhanced by cellular or exogenous heparin/HS (Terman, Khandke et al. 1994; Gitay-Goren, Cohen et al. 1996) suggesting that HS on HSPG regulate the interaction of VEGF to VEGFR and consequent biological activity (Schlessinger, Lax et al. 1995).

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CHAPTER II
ANTISENSE-MEDIATED SUPPRESSION OF *HEPARANASE* GENE INHIBITS MELANOMA CELL INVASION*

INTRODUCTION

Mechanisms responsible for melanoma progression to highly aggressive metastatic disease are not fully understood. However, it is known that proteolytic enzymes play important roles due to their involvement in extracellular matrix (ECM) disassembly, which allows tumor cells to invade into distant organs. Heparan sulfate proteoglycans (HSPG) (Iozzo 2001) are now recognized as cell surface/ECM active biologic modulators (Kjellen and Lindahl 1991), and their degradation at the level of heparan sulfate glycosaminoglycan chains (HS) has significant regulatory consequences in cancer metastasis. Elevated levels of heparanase (HPSE-1) are known to be associated with brain-metastatic melanoma (Marchetti, Menter et al. 1993; Marchetti, McQuillan et al. 1996; Marchetti and Nicolson 2001; Marchetti 2002; Marchetti 2002). HPSE-1 is an endo-β-D-glucuronidase (Nakajima, Irimura et al. 1988) involved in the degradation of cell surface/ ECM HSPG of a wide range of normal and neoplastic tissues (Hulett, Freeman et al. 1999; Kussie, Hulmes et al. 1999; Toyoshima and Nakajima 1999; Vlodavsky, Friedmann et al. 1999; Sasaki, Higashi et al. 2004) and a molecular determinant of metastatic events. The enzymatic activity of HPSE-1 is characterized by specific intrachain HS cleavage of glycosidic bonds with a hydrolase (but not eliminase) type of action, therefore facilitating the release of several protein modulators of cell function, including migration, adhesion, inflamm-

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ation, angiogenesis, embryogenesis, invasion and metastasis (Parish, Coombe et al. 1987; Nakajima, Irimura et al. 1988; Vlodavsky, Bar-Shavit et al. 1991; Hulett, Freeman et al. 1999; Kussie, Hulmes et al. 1999; Toyoshima and Nakajima 1999; Vlodavsky, Friedmann et al. 1999; Vlodavsky and Friedmann 2001; Zetser, Bashenko et al. 2003; Sasaki, Higashi et al. 2004). Secondly, HPSE-1 levels have been found to be increased in sera and urine of human patients with metastatic cancers (Parish, Coombe et al. 1987; Nakajima, Irimura et al. 1988; Vlodavsky, Bar-Shavit et al. 1991; Hulett, Freeman et al. 1999; Kussie, Hulmes et al. 1999; Toyoshima and Nakajima 1999; Vlodavsky, Friedmann et al. 1999; Vlodavsky, Goldshmidt et al. 2001; Kelly, Miao et al. 2003; Zetser, Bashenko et al. 2003; Sasaki, Higashi et al. 2004). Thirdly, HPSE-1 activity correlates with the metastatic potential of tumor cells in animal models, resulting in increased mortality (Vlodavsky, Goldshmidt et al. 2001). Finally, enhanced HPSE-1 mRNA levels correlate with reduced postoperative survival in cancer patients (Vlodavsky, Bar-Shavit et al. 1991; Hulett, Freeman et al. 1999; Kussie, Hulmes et al. 1999; Vlodavsky, Friedmann et al. 1999; Vlodavsky, Goldshmidt et al. 2001; Sasaki, Higashi et al. 2004).

HPSE-1 from various mammalian sources has been cloned as a single gene family (Hulett, Freeman et al. 1999; Kussie, Hulmes et al. 1999; Toyoshima and Nakajima 1999; Vlodavsky, Friedmann et al. 1999; Sasaki, Higashi et al. 2004), representing the dominant HS-degradative enzyme. Importantly, HPSE-1 regulation has been shown to change metastatic properties of tumors (Vlodavsky, Friedmann et al. 1999; Uno, Fujiwara et al. 2001; Zhang, Fu et al. 2003). For example, an upregulation of the enzyme has been shown to increase the metastatic properties of tumor cells (Vlodavsky, Friedmann et al. 1999; Uno, Fujiwara et al. 2001). Conversely, downregulating HPSE-1 by antisense strategies (Uno, Fujiwara et al. 2001; Zhang, Fu et al. 2003) demonstrated a reduction in the invasive properties of neoplastic cells.

Evidence has also demonstrated that HPSE-1 is a potential target for antimetastasis drugs because of its critical roles in angiogenic and invasive processes: treatment of experimental animals with HPSE-1 inhibitors considerably reduced the incidence of tumor invasion and angiogenesis in animal models (Parish, Coombe et al. 1987; Marchetti, Reiland et al. 2003). Extensive available animal and clinical data suggest that HPSE-1 may play an important role in the progression of a variety of human tumors. Nevertheless, the possible role(s) of HPSE-1 in tumor progression at the molecular and cellular levels is poorly understood.

These observations led us to hypothesize that inhibition of HPSE-1 expression could inhibit tumor cell invasion by metastatic melanoma cells. Here we demonstrate that an adenoviral vector expressing the full-length human heparanase gene in an antisense orientation specifically inhibits HPSE-1 expression as well as its invasiveness, leading to a reduction of invasive capabilities by metastatic melanoma cells *in vitro* and *in vivo*. Our results further support the fact that inhibiting HPSE-1 can change the invasive properties of melanoma cells like others have found in lung or breast carcinoma cell lines.

MATERIALS AND METHODS

Materials

Heparan sulfate (HS) from bovine kidney was acquired from Sigma Chemical Company (St. Louis, MO). DMEM and Ham's F-12 nutrient medium and trypsin-EDTA were purchased from Gibco (Grand Island, New York, NY), and FBS from Hyclone Laboratories (Logan, UT). Human recombinant HPSE-1 was kindly provided by Dr. Edward McKenzie (McKenzie, Young et al. 2003). The polyclonal antibodies to human HPSE-1 were generously provided by Dr. Laurie A. Dempsey (Mayo Clinic, Rochester, Minnesota) and Dr. Robert L. Heinrikson (Pharmacia-Upjohn Inc., Kalamazoo, Michigan).

Transwell cell culture chambers were purchased from Corning Incorporated Life Sciences (Acton, MA), while Matrigel™ was obtained from BD Biosciences Discovery Labware (Bedford, MA) and fibronectin from ICN Biochemical (Irvine, CA). All other chemicals used were reagent grade or better.

Cells and Tissue Culture Conditions

Early-passage melanoma cells with varying metastatic abilities, both of murine (B16B15b line (Nakajima, Irimura et al. 1983) and human origin (70W line) (Ishikawa, Dennis et al. 1988; Ishikawa, Fernandez et al. 1988) were maintained as monolayer cultures in a 1:1 (v/v) mixture of DMEM/F-12 supplemented with 10% (v/v) FBS and 5 mM sodium butyrate (B16B15b) or 10% (v/v) FBS (70W). B16B15b and 70W metastatic melanoma cells (lung being the primary colonization site) were chosen as a source of HPSE-1 because they are highly invasive and produce HPSE-1 at elevated levels versus their respective parental counterparts (Marchetti, Menter et al. 1993; Marchetti, McQuillan et al. 1996). 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 (70W) before reaching confluence. The transformed embryonic kidney cell line 293 was grown in DMEM/F-12, supplemented with 10% FBS and penicillin (100 units/ml), and streptomycin (100 μg/ml). The 293 cells were used for the production of adenoviral vectors (Uno, Fujiwara et al. 2001).

Construction of Recombinant Adenovirus Containing the Human *Heparanase* Gene

An adenovirus expression vector kit (Takara Biomedicals Inc., Tokyo, Japan) was used to generate recombinant adenovirus for the expression of human HPSE-1 gene in both sense and antisense orientations. Replication-deficient, E1- and E3-deleted recombinant adenovirus serotype 5 (Ad5) was used as the viral backbone. Plasmid DNA containing the cloned *HPSE-1*

gene and synthetic oligonucleotides Hep-5' (complementary to the 5' end of the gene) and Hep-3' (complementary to the 3' end of the gene) were used to produce PCR products encompassing the HPSE-1 sequence (1632 bp). PCR products were treated with T4 DNA polymerase to generate uniformed blunt ends required for the ligation reaction into pAxCAwt cosmid provided by the Takara kit. After cloning the HPSE-1 gene into cosmid vector, availability of the recombinant cosmids containing the target gene in sense (5' - 3' under the CAG promoter of the vector) and antisense (3' - 5') orientations were confirmed by restriction analysis. Cosmid DNAs were produced in large quantities and after gradient purification were used for cotransfection with adenovirus genomic DNA-terminal protein complex (DNA-TPC provided by the kit) into 293 cells. Following the kit protocol, recombinant adenoviruses expressing human HPSE-1 in both sense (Ad-S/hep) and antisense (Ad-AS/hep) orientations were generated. Integrity of these recombinant viruses was confirmed by PCR and restriction analysis. The E1-, E3-deleted, replication deficient adenovirus pAd5-Blue was used as the control vector. Viral titer was quantified by determination of the 50% infectivity on tissue culture (TCID 50) in 293 cells.

Adenoviral Infection

70W and the B16B15b cells were plated on 100 mm dishes (10^6 cells/dish). Twenty four hours later, cells were washed twice with PBS containing 2 mM EDTA, and infected with virus diluted in serum-free DMEM/F-12. To maximize cell viability and protein expression, cells were infected with the viral vectors at a multiplicity of infection (M.O.I.) of 30-50. Plates were then incubated at 37°C for 1 hr, rocking gently every 10 min. Infection was terminated by adding culture medium containing penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells

were then incubated at 37°C for an appropriate period of time. Immunofluorescent control experiments were performed to confirm cell infectivity of our adenoviral vectors.

Reverse Transcription (RT) PCR Analysis

Total RNA was isolated from cells using RNeasy kit (Qiagen Inc., Valencia, CA). Briefly, reverse transcription was performed at 42°C for 1 hour, and PCR was performed using specific primers (25 µl final volume). The following specific primers were used: HPSE-1 sense (HPSE-1-S: 5'-TTC GAT CCC AAG AAG GAA TCA AC-3') and HPSE-1 antisense (HPSE-1-AS: 5'- TAC ATG GCA TCA CTA C -3'); for the sequence just after the inserted fragment in the vector (Ad5 pax: 5'-ATC GAT TCT AGA CTA GTT TAA TTA ATT T-3'); control glyceraldehyde-3-phosphate dehydrogenase, sense (5'- TGA AGG TCG GAG TCA ACG GAT TTG GT -3') and antisense (5'- CAT GTG GGC CAT GAG GTC CAC CAC -3'). The amplification reaction for HPSE-1, Ad-S/hep and Ad-AS/hep specific constructs involved 35 cycles of denaturation at 95°C for 45 seconds, annealing at 52°C for 1 min, and 72°C for 1 min. The amplification for GAPDH reaction involved 35 cycles of denaturation at 94°C for 30 seconds and annealing at 68°C for 3 min in the GeneAmp PCR-system 9700 (Applied Biosystems, Foster City, CA). PCR products were subsequently resolved on 1% agarose gels and visualized by ethidium bromide staining.

Western Blot Analysis

Cells were plated (10^6 cells/plate) on 100 mm dishes and allowed to attach and grow for 24 hr. Cells were subsequently infected with Ad-S/hep, Ad-AS/hep or pAd5-Blue control vector and incubated for 36-48 hours. They were then released and centrifuged (300 rpm for 5 min) and resuspended in lysis buffer [TBS (pH 7.4) containing Triton-X 100 (0.5%), leupeptin (10 µg/ml), pepstatin (10 µg/ml) and PMSF (0.2 mM)] at a density of 10^8 cells/ml. Cells were subsequently

vortexed and kept on ice for 10 min followed by centrifugation for 10 min at 13,000 rpm at 4°C. Supernatant was collected and protein concentration was determined by the BCA assay. Protein samples (60-90 μg) were then incubated at 100°C for 10 min with Laemmli sample buffer (Laemmli 1970) and separated on 10% Criterion gels (Tris-HCl, Bio-Rad Laboratories, Hercules, CA). Gels were transferred to a PVDF membrane (Pierce Endogen Inc., Rockford, IL) and incubated overnight (18 hrs) in a blocking reagent [3% (w/v) non-fat dry milk, 0.5% (w/v) BSA, 0.3% (v/v) Tween-20 in PBS, pH 7.5]. HPSE-1 protein was labeled using anti-HPSE-1 PAb (1: 5,000 dilution) (Mayo Clinic, Rochester, Minnesota) in 3% (w/v) non-fat dry milk, 0.5% (w/v) BSA, washed for 1 hr with 6-8 changes of 0.5% IGE-PAL (CA-630, Sigma Chemical Company, St. Louis, MO) in Tris buffer (20 mM Tris, 150 mM NaCl, pH 7.4), and then incubated with horseradish peroxidase-anti-rabbit IgG (1: 5,000 dilution; Accurate Scientific, Westbury, NY). It was subsequently washed and developed using the Super-signal west femto maximum sensitivity substrate (Pierce Endogen Inc., Rockford, IL). Dual-Color Precision Plus Protein Standards (Bio-Rad Laboratories Inc., Hercules, CA) were used as molecular weight markers. Labeling was detected and quantified using a Versadoc imaging system (Quantity One, Bio-Rad Laboratories Inc., Hercules, CA).

HPSE-1 Activity Assays

HPSE-1 activity was determined by degradation of FITC-HS using high-speed gel permeation column chromatography (HPLC) as previously described (Toyoshima and Nakajima 1999; Reiland, Sanderson et al. 2004). Briefly, cell-lysates (80 μg cell lysate/μg of HS) were prepared as described above and incubated with FITC-HS at 37°C for 18 hr in 100 □l of sodium acetate (100 mM, pH 4.2). Reaction was terminated by heating samples for 100°C for 15 min. HS products yielded by HPSE-1 reaction were analyzed by size exclusion chromatography

performed on a HP1090 liquid chromatograph with a photodiode array detector (Agilent Technologies Inc., Wilmington, DE) and a HP1046 programmable fluorescence detector (Agilent Technologies Inc., Wilmington, DE). Samples (25 µl) were injected on a TSK-GEL 3000SW_{XL} column (5 µm, 7.8 mm x 30 cm) with a TSK-GEL SW_{XL} guard column (5 µm, 6.0 mm x 4.0 cm) from Tosoh Bioscience (Montgomeryville, PA) at an ambient temperature (25^{o}C). Samples were isocratically eluted using Tris-HCl (25 mM), NaCl (150 mM), pH 7.5 at a flow rate of 0.5 ml/min with a sample splitter placed between the column and detectors to maintain a column pressure of less than 70 bar. Fluorescence was monitored with excitation at 490 nm and emission at 520 nm, then data was processed and peaks were integrated using HP ChemStation software (Agilent Technologies Inc., Wilmington, DE). HPSE-1 activity was determined by measuring the decrease in fluorescence intensity in the first one-half area of the intact FITC-HS peak chromatogram. The retention times were calculated from the highest peak of the chromatograms.

Alternatively, a commercial heparan degrading enzyme assay kit (Takara Mirus Biomedical Inc., Madison, WI) was used to determine HPSE-1 activity in melanoma cells (Reiland, Sanderson et al. 2004). Indicated amounts of cell-lysate were incubated with biotinylated-HS at 37^{o}C for 45 min and HPSE-1 activity determined by an ELISA type assay. Color was developed using the substrate supplied in the kit and plates were read at 450 nm using a microplate reader (EL 808, Bio-Tek Instruments Inc., Winooski, VT).

Chemoinvasion Assays

Invasive properties of melanoma cells were assayed by Boyden's chambers as previously described (Marchetti, Menter et al. 1993). Briefly, cell invasion was assayed using Transwell cell culture chambers (12 µm pore size, 12 mm diameter) coated with Matrigel^{TM} (diluted as 38

μg /ml in cold DMEM/F-12 with P/S, 100 μl final coating volume) that was applied to the upper filter surface and allowed to dry before use. Cells were plated on day 1 and after 24 hours were infected with Ad-S/hep, Ad-AS/hep or with the control vector pAd5-Blue as described above. Twenty four hours later, cells were released from the culture plate and added to the upper chamber of invasion plates (1.2 x 10^5 cells/chamber) in serum-free DMEM/F-12. Lower chambers contained n-formyl-l-methyl-l-leucine-l-phenylalanine (5 nM) in DMEM/F-12 with 10% (v/v) FBS and fibronectin (1 μg/ml) as chemoattractants. Cells were incubated in invasive chambers for 48-72 hrs at 37°C in a humidified 5% CO_2/95% air (v/v) atmosphere. At the end of the experiment, tranwell membranes were lifted from the wells carefully without touching the bottom. Non-invasive cells were wiped from the upper chamber with a cotton swab and invasive cells were visualized with Diff-Quick Stain Kit (IMEB Inc., San Marcos, CA) according to manufacturer's instructions. Briefly, membranes were dipped in solution A (methanol) for 2 minutes and allowed to air dry. Membranes were then transferred in solution B (Eosin), dipped 25 times, transferred in solution C (Azure), and then dipped 25 more times. After rinsing with deionized water, membranes were inverted and allowed to dry. By using this kit, the nuclei of cells are stained blue and cell cytoplasm is stained pink. The number of invasive cells was obtained by counting the nuclei under the microscope.

***In Vivo* Tumorigenic Assays**

Human malignant melanoma 70W cells were infected with Ad-S/hep, Ad-AS/hep and control pAd5-Blue at a M.O.I. of 50. Cells were harvested 36 hrs after infection, and resuspended in DMEM/F-12 at a density of 5.0 x 10^6 cells/ml. 6-weeks old female athymic *nu/nu* mice were injected I.V. (tail vein) with 200 μl of cell suspension (10^6 cells) through a 27-gauge needle. Mice were euthanized and examined for tumor formation as previously described

(Ishikawa, Fernandez et al. 1988). Metastatic behavior per animal group (number of lung nodules and lungs wet weight; N = 15) was then determined. Additionally, lung tumor tissues were saved in formalin and random samples were taken and stained with hematoxylin and eosin and analyzed under microscopy.

Immunohistochemical Analysis

Immunohistochemical staining was performed using a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) according to the instructions provided by the manufacturer. Formalin-fixed, paraffin-embedded tissue sections from *in vivo* tumorigenic assays were mounted on silanized slides and deparaffinized. Endogenous peroxidase was blocked by incubating the sections in 3.0% H_2O_2 without pretreatment. After the blocking of nonspecific reactivity with rabbit serum for 30 min at room temperature (25^{o}C), sections were incubated at 4°C for 60 min. with antihuman HPSE-1 rabbit polyclonal antibody raised against human heparanase (Pharmacia-Upjohn Inc., Kalamazoo, Michigan). Following rinsing, slides were incubated with biotinylated antirabbit IgG and then with Vectastain ABC reagent. Peroxidase activity was determined using nova red and counterstained with hematoxylin. As a negative control, sections were subjected to normal serum blocking with omission of the primary antibody.

RESULTS

Detection of HPSE-1 mRNA Expression in Sense or Antisense Orientation

To examine the significance of human heparanase in metastatic melanoma cell invasion, we constructed replication-defective recombinant adenoviruses expressing sense and antisense RNA to a full-length human HPSE-1 sequence, Ad-S/hep and Ad-AS/hep, respectively. Both sense and antisense transcripts of HPSE-1 could be detected when HPSE-1-S and HPSE-1-AS

primers were used in 70W cells, but not in the B16B15b cells since primers chosen against human *heparanase* did not amplify the murine *heparanase*. Strand-specific primer pairs (HPSE-1-S and Ad5pax, or HPSE-1-AS and Ad5pax) detected the expression of the sense and antisense constructs in B16B15b and 70W melanoma cells with Ad-S/Hep or Ad-AS/hep treatment respectively (Figure 2.1).

Modulation of HPSE-1 Protein Levels Following Ad-S/Ad-AS/hep Infection in Melanoma Cells

Western blot analyses were performed to examine the effects of Ad-S/hep and Ad-AS/hep infection on HPSE-1 protein levels in B16B15b and 70W cells. Since HPSE-1 is a low-abundance protein, 60-90 μg of total cell-lysate had to be electrophoresed in order to detect adequate HPSE-1 levels (Figure 2.2). HPSE-1 protein expression increased with Ad-S/hep and decreased with Ad-AS/hep treated melanoma cells compared to treatment with control vector pAd5-Blue in both B16B15b (Figure 2.2A) and 70W (Figure 2.2B) cells. HPSE-1 is a heterodimer consisting of a 50kDa and a 8kDa band processed from a pro-enzyme precursor (65kDa) by proteolytic cleavage and, presence of the 8kDa band is essential for its enzymatic activity (Fairbanks, Mildner et al. 1999; Hulett, Freeman et al. 1999; Kelly, Miao et al. 2003). We detected the active form of HPSE-1 in the cells; however it migrated slightly slower than its predicted molecular weight (50 kDa). This is consistent with the recent findings by Simizu et al. (Simizu, Ishida et al. 2004), and possibly due to post-translational modification in the glycosylation of the enzyme. We also detected a weak band representing the 65kDa inactive precursor in B16B15b cells. Furthermore, we found a two-fold increase in HPSE-1 protein level using the Ad-S/hep vector and a 30% decrease with the Ad-AS/hep in the B16B15b cell line (Figure 2.2A). In 70W cells, we observed a 30% increase or decrease in HPSE-1 protein by densitometric analysis following Ad-S/hep or Ad-AS/hep treatment, respectively (Figure 2.2B).

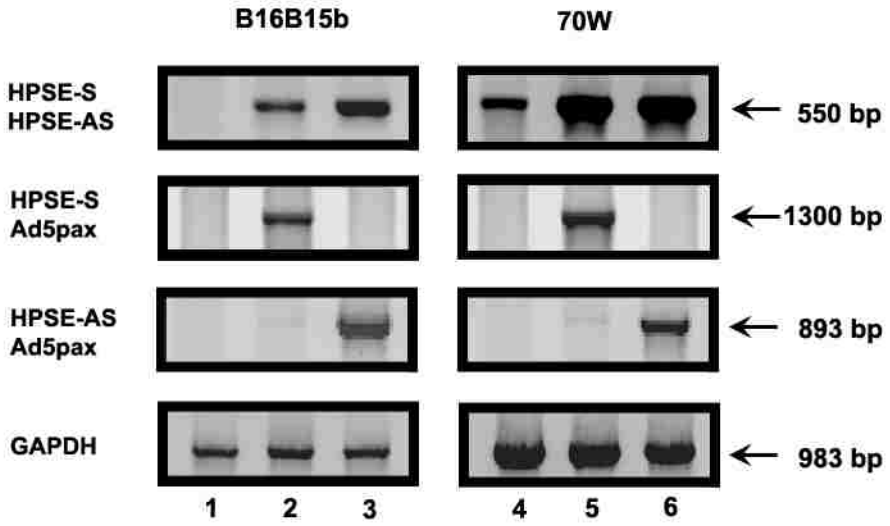

Figure 2.1. Expression of human *heparanase* sense and antisense transcripts in melanoma cells. B16B15b and 70W cell lines were infected with pAd5-Blue (lanes 1 and 4), Ad-S/hep (lanes 2 and 5), or Ad-AS/hep vector (lanes 3 and 6) and subjected to RT-PCR analysis 36 hours after infection. Two primer pairs, HPSE-1-S/Ad5 pax and HPSE-1-AS/Ad5pax, were designed to, respectively, detect sense and antisense transcripts of the human heparanase gene. Both sense and antisense transcripts of HPSE-1 could be detected when HPSE-1-S and HPSE-1-AS primers were used in 70W cells, but not in the B16B15b cells because the primers chosen against human heparanase gene did not amplify murine heparanase. Primers and predicted products are indicated at the side of panel.

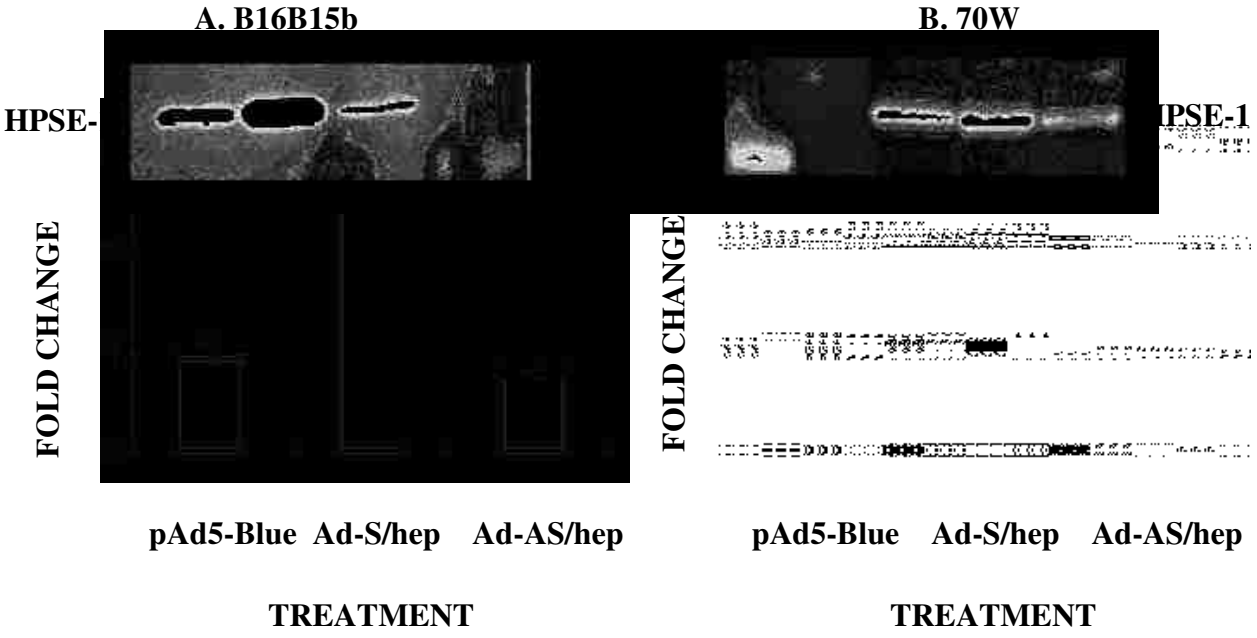

Figure 2.2. Western blot analysis in melanoma cells for HPSE-1 protein expression. Cells (B16B15b and 70W) were infected with pAd5-Blue (lanes 1 and 4), Ad-S/hep (lanes 2 and 5), or Ad-AS/hep (lanes 3 and 6) (see Experimental Procedures section) and analyzed for HPSE-1 expression after 48 hours. Equal amounts (60 – 90 µg) of protein were loaded on gels and HPSE-1 protein levels were detected by a rabbit polyclonal antibody and HRP– antirabbit IgG followed by use of the Supersignal west femto maximum sensitivity substrate. Bands were visualized on a Versadoc imaging system. Quantification was performed using Quantity One software program (Bio-Rad Laboratories Inc.). (A) Murine B16B15b. (B) Human 70W.

HPSE-1 Activity is Upregulated in Ad-S/hep–Treated Melanoma Cells

We used a specific HPSE-1 activity assay on fluorescein isothiocyanate (FITC)–labeled HS in 70W cells. This assay detects HPLC profile shifts based on the size of HS fragments (Toyoshima and Nakajima 1999; Reiland, Sanderson et al. 2004). Since HPSE-1 cleaves the HS chains into discrete fragments, we detected a profile shift when compared to FITC-HS alone which was used as negative control (Toyoshima and Nakajima 1999; Reiland, Sanderson et al. 2004) (data not shown). We observed a decrease in fragment size in Ad-S/hep-treated 70W cells compared to mock-infected cells. We did not observe a significant difference between Ad-S/hep and mock-treated cells since this represents a small shift compared to the total profile area (Figure 2.3A). However, we observed a significant difference in their respective HPLC retention times which detect changes in the size of predominant HS fragments ($p < 0.02$). This analysis is more sensitive to small changes in HS fragment size and showed less activity with Ad-AS/hep-treated cell lysates (Figure 2.3B). The Ad-S/hep-treated cell lysates showed a higher retention time compared to Ad-AS/hep or mock treatment suggesting that increased digestion of HS chains resulted from an augmented HPSE-1 activity following Ad-S/hep treatment in the 70W cell line ($p< 0.05$). We also performed the ELISA-type assay and found similar changes of HPSE-1 activity in response to pAd5-Blue, Ad-S/hep, or Ad-AS/hep treatments (Table 2.1).

Secondly, we measured HPSE-1 activity in B16B15b and 70W cells co-infected with Ad-S/hep and Ad-AS/hep, or pAd5-Blue vectors using biotinylated HS and an ELISA kit. We detected a dose-dependent inhibition of HPSE-1 activity with a higher dose of Ad-AS/hep vector (Table 2.2).

Next, we used suramin, a HPSE-1 antagonist, to inhibit the activity of Ad-S/hep infected cells (Figure 2.4). This suggests that 1) the increased activity (Figure 2.4A) and retention time

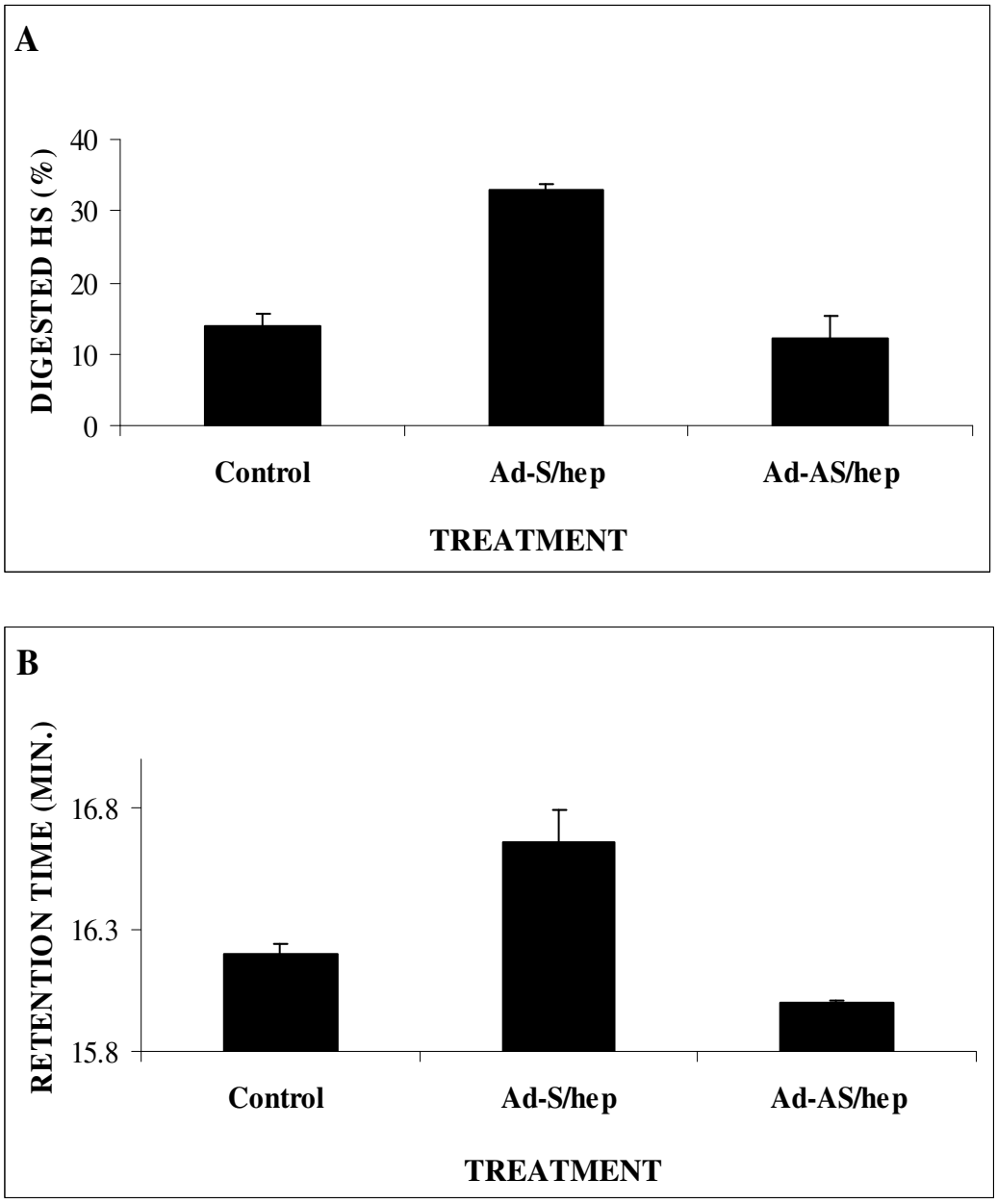

Figure 2.3. HPSE-1 activity increases with Ad-S/hep treatment in 70W cells compared to mock-treated cells. Cells were infected with Ad-S/hep, Ad-AS/ hep, or none, and analyzed for HPSE-1 activity after 48 hours. Equal amounts (100 µg) of cell lysates were incubated with FITC-HS for 18 hours (see Experimental Procedures section) and run on HPLC to analyze HPSE-1 activity. (A) Percentage of HS digested with different treatments. (B) Retention times calculated with different treatments. Bars represent the mean with standard deviation of triplicate determinations.

Table 2.1. Modulation of HPSE-1 activity with Ad-S/hep or Ad-AS/hep treatment

Treatment (MOI)	A_{450} (nm)*
pAd5-Blue	0.65 ± 0.05
Ad-S/hep	0.21 ± 0.03
Ad-AS/hep	0.73 ± 0.06

* The higher the OD value, the lower is the HPSE-1 activity. The A_{450} starting point of no enzyme degradation was 0.79.

Table 2.2. HPSE-1 activity decreases with Ad-AS/hep treatment in a dose-dependent manner

Cell line	Treatment (MOI)			A_{450} (nm)*
	pAd5-Blue	Ad-S/hep	Ad-AS/hep	
B16B15b	-	-	-	1.1 ± 0.19
	125	-	-	1.0 ± 0.14
	100	25	-	0.87 ± 0.01
	50	25	50	0.92 ± 0.03
	-	25	100	1.2 ± 0.10
70W	-	-	-	0.90 ± 0.33
	125	-	-	0.83 ± 0.04
	100	25	-	0.59 ± 0.01
	50	25	50	0.72 ± 0.02
	-	25	100	0.87 ± 0.16

* The higher the OD value, the lower is the HPSE-1 activity. The A_{450} starting point of no enzyme degradation was 1.7.

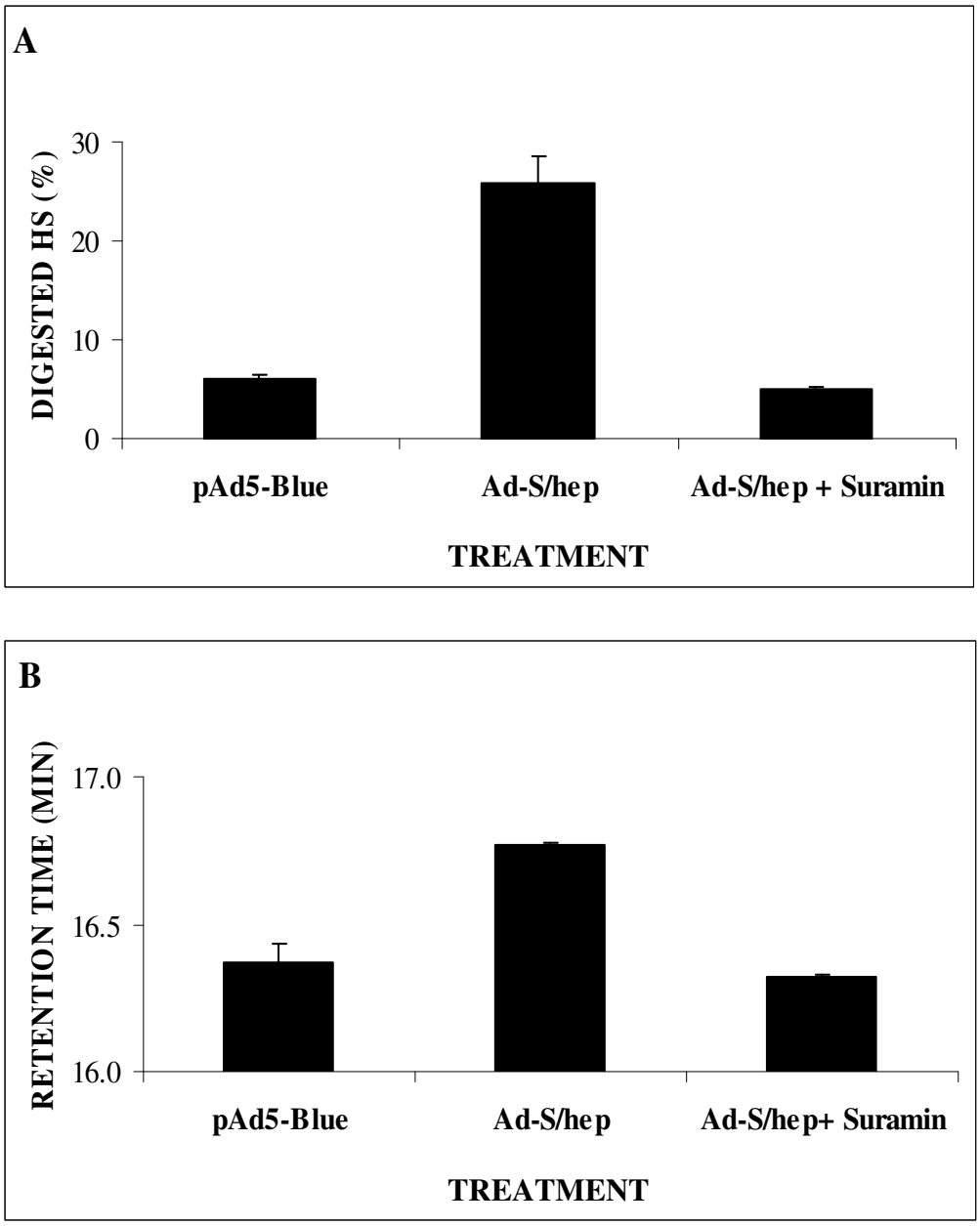

Figure 2.4. HPSE-1 activity is inhibited by suramin in Ad-S/hep – treated 70W cells. Cells were infected with Ad-S/hep or pAd5-Blue as control and analyzed for HPSE-1 activity after 48 hours. Equal amounts of cell lysates were incubated with FITC-HS for 18 hours with or without suramin (500 μM) and run on HPLC to analyze HPSE-1 activity. (A) The percentage of HS digested with different treatments. (B) Retention times calculated with different treatments. Bars represent the mean with standard deviation of triplicate determinations.

(Figure 2.4B) in the assay is due to an up-regulation of HPSE-1 in the Ad-S/hep - infected cells, and 2) this activity can be further modulated by suramin (500 μM) as an HPSE-1 inhibitor (Figure 2.4).

Inhibition of HPSE-1-Induced Invasion by Ad-AS/hep Infection of Melanoma Cells

To investigate HPSE-1 mechanisms in metastatic cell invasion, chemoinvasion assays were performed using Matrigel^{TM}- coated Transwell chamber systems. B16B15b and 70W cells when infected with Ad-S/hep, possessed augmented invasive properties compared with pAd5-Blue-infected cells, while Ad-AS/hep - treated cells showed a significant inhibition of invasion (Figure 2.5). These results suggest that the antisense adenoviral construct selectively inhibited HPSE-1 - induced invasive properties in B16B15b and 70W cells.

Inhibition of Tumor Formation with Ad-AS/hep - Treated 70w Cells

To evaluate HPSE-1 mechanism in metastatic cell invasion, human malignant melanoma 70W cells were infected with Ad-S/hep, Ad-AS/hep or pAd5-Blue vectors and injected in 6-weeks old female athymic nude (*nu/nu*) mice. Mice injected with pAd5-Blue and Ad-S/hep - treated 70W cells developed tumors but none of the Ad-AS/hep - treated cells were capable of extensive tumor formation (Figure 2.6, Table 2.3). During quantitation of tumor formation, we found microscopic tumors in one of the mice treated with Ad-AS/hep suggesting that the antisense adenoviral construct selectively inhibited HPSE-1-induced invasive properties in 70W cells. Because control cells already expressed relatively high levels of HPSE-1, we were unable to see a significant difference between our control and Ad-S/hep groups; however, we found a significant difference between the control and the Ad-AS/hep groups. Furthermore, formalin-fixed tissues from each group were analyzed for immunohistochemistry (Figure 2.7): lung tumor tissue from the Ad-S/hep group expressed HPSE-1 more consistently and with an increased

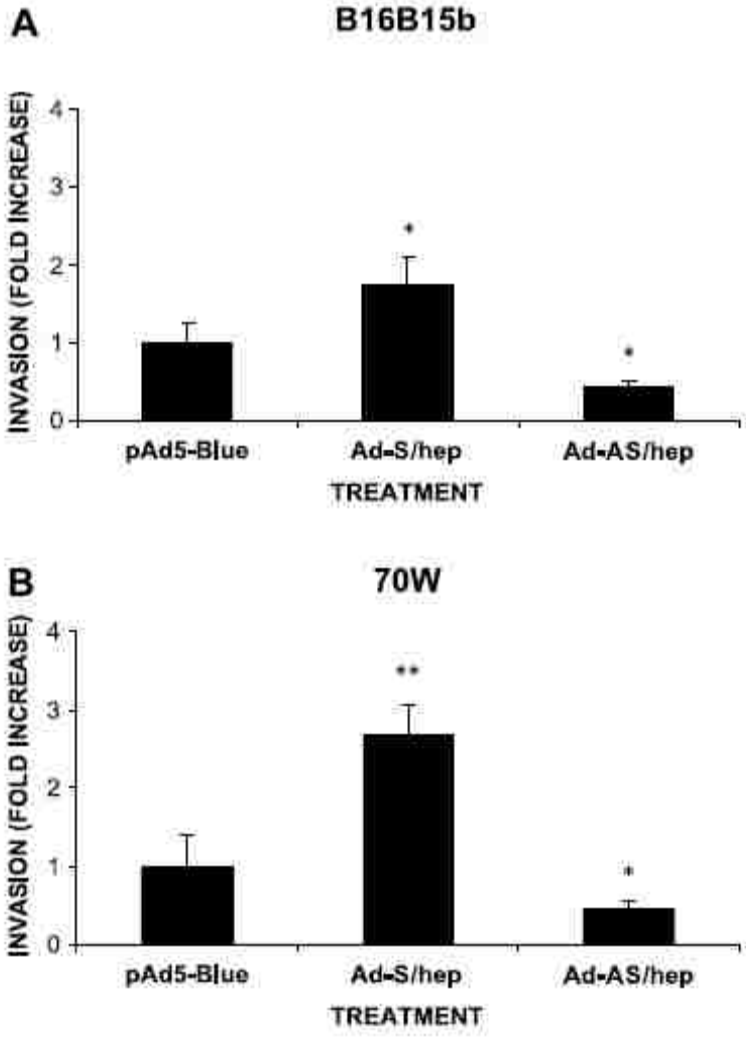

Figure 2.5. Ad-AS/hep treatment inhibits HPSE-1–mediated invasion of melanoma cells. Metastatic melanoma cells (B16B15b and 70W) infected with pAd5-Blue, Ad-S/hep, or Ad-AS/hep were placed in invasion chambers (12 µm diameter pore size) for 72 hours at 37^0C in a humidified 5% CO2/95% air (vol/vol) atmosphere. Noninvasive cells were wiped from the upper chamber with a cotton swab and invasive cells were detected with the Diff-Quick Stain Kit. Bars represent the mean with standard deviation of triplicate determinations (*$P < 0.01$, **$P < 0.1$). Student's t test was used as statistical method.

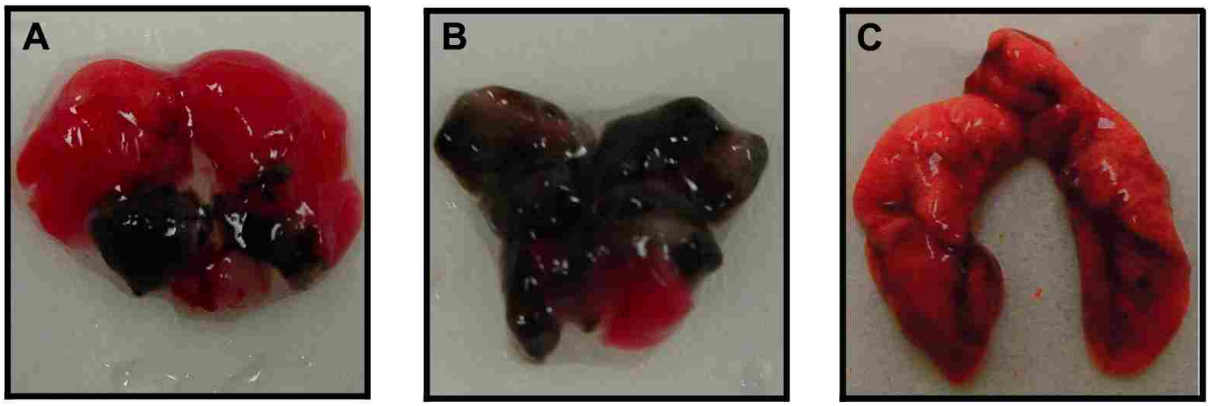

Figure 2.6. Ad-AS/hep treatment inhibits tumor formation in athymic nude (*nu/nu*) mice. Human malignant melanoma 70W cells were infected with pAd5-Blue control vector (A), Ad-S/hep (B), or Ad-AS/hep (C) and were injected intravenously in 6-week – old female athymic nu/nu mice. At the end of the experimental period, mice were euthanized and examined for tumor formation. Quantification of lung tumor formation and statistical analyses were performed on the number of experimental animals and shown in Table 2.3.

Table 2.3 - Ad-AS/hep treatment inhibits tumor formation in nude (*nu/nu*) mice

Treatment	Number of Lung Tumor Nodules		Lung Weight
	Median	Range	(mg, mean ± SD)
pAd5-Blue	16	0-32[a]	571 ± 154
Ad-S/hep	12	0-44[b]	453 ± 210
Ad-AS/hep	0	0-4[c]	366 ± 75

a: Eighty percent of mice had lung tumors. *b:* Eighty percent of mice had lung tumors. *c:* Twenty percent of mice had lung tumors ($p = 0.04$). Student's t test was used as statistical method.

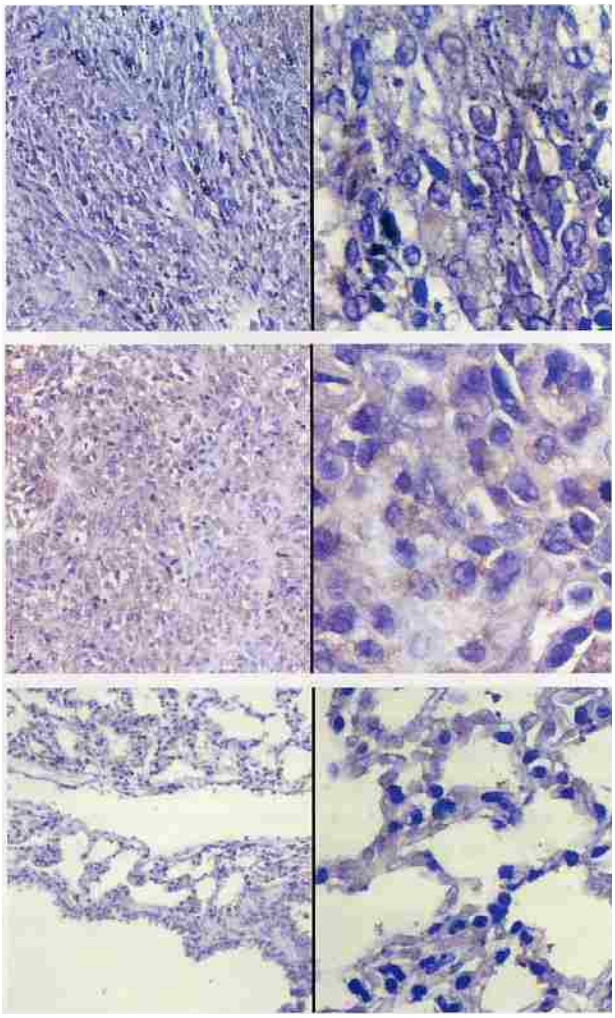

Figure 2.7. Ad-S/hep/Ad-AS/hep–mediated HPSE-1 expression in nude (*nu/nu*) mice. Animals were injected with pAd5-Blue – , Ad-S/hep – , or Ad-AS/hep – infected 70W cells and formalin-fixed lung tumor tissues were analyzed by immunohistochemistry. Intense staining of HPSE-1 was found in lung tissues of animals treated with Ad-S/hep – infected 70W cells (C and D) compared to pAd5-Blue (A and B) or Ad-AS/hep (E and F) treatment. HPSE-1 staining observed in the Ad-AS/hep is due to the presence of the enzyme in the endothelium and macrophages in lung tissues. Digital images were produced on an Axioplan microscope with advanced spot imaging program at x10 (A, C, and E) and x40 (B, D, and F) objectives using identical conditions for all photographs and antibodies used; x40 objectives show the different intensities of HPSE-1 staining by various treatments.

immunohistochemical intensity than in the control group. HPSE-1 staining was also observed in the Ad-AS/hep group but mainly due to presence of the enzyme in the endothelium (Marchetti 1997) and macrophages (Sasaki, Higashi et al. 2004) of lung tissues (Figure 2.7).

DISCUSSION

Invasion and metastasis are characteristic features of malignant tumors and are among the greatest impediments to curing cancer. Inhibition of tumor invasion is an attractive approach for the treatment of highly malignant tumors (Zetter 1998; Folkman 2001). Substantial evidence accumulated over the last three decades indicates that HSPG act to inhibit invasion by promoting tight cell–cell and cell–ECM interactions (Bernfield, Gotte et al. 1999; Iozzo 2001; Sanderson 2001). Degradation of HS weakens cell–cell and cell–matrix adhesion in melanoma and other tumor cells (Dhodapkar, Kelly et al. 1997; Ma and Geng 2000; Engbring, Hoffman et al. 2002). HSPG can also act both as reservoirs of growth factors (Iozzo, Cohen et al. 1994; Vlodavsky and Friedmann 2001) and as coreceptors for ligand binding and subsequent intracellular signaling. These heparin-binding factors are involved in growth, invasion, angiogenesis, and tumor progression (Bernfield, Gotte et al. 1999; Iozzo and San Antonio 2001; Vlodavsky, Goldshmidt et al. 2001). HS-degradative enzymes, such as heparitinases, heparinases from Flavobacterium heparinum, or endoglucosaminidases, cleave HS to disaccharides and tetrasaccharides, which are too short for growth factor and ECM ligand binding (Ernst, Langer et al. 1995; Ernst, Venkataraman et al. 1996; Liu, Shriver et al. 2002). However, HPSE-1 is an endo-β-D-glucuronidase that cleaves HS at specific intrachain sites, resulting in fragments of appreciable size (10–20 sugar units) (Nakajima, Irimura et al. 1984; Nakajima, Irimura et al. 1988; Marchetti 1997; Vlodavsky, Goldshmidt et al. 2001). This confirms the notion that HPSE-1, in degrading HSPG and releasing HS-bound angiogenic/growth factors, may aid the modulation of growth

factor activities in metastasis (Marchetti, Denkins et al. 2003; Reiland, Sanderson et al. 2004). Thus, HPSE-1 is an attractive target for the development of novel antimetastatic drugs because of the considerable evidence implicating this enzyme in tumor cell invasion (Vlodavsky and Goldshmidt 2001).

In the present study, we have demonstrated that overexpression of human HPSE-1 enhanced tumor cell invasiveness *in vitro* and *in vivo*, and that this invasive ability was significantly reduced by inhibiting HPSE-1 expression using an adenovirus-mediated antisense gene delivery strategy. HPSE-1 expressions, as well as its biologic activity, were enhanced in metastatic melanoma cell lines following Ad-S/hep treatment. Conversely, treatment with Ad-AS/hep resulted in decreased HPSE-1 expression as well as cell invasiveness *in vitro* and *in vivo*. For example, in our experiments designed to analyze the effects of Ad-S/hep, we found that there was an upregulation of protein expression in both B16B15b and 70W melanoma cell lines. Conversely, we observed the opposite using Ad-AS/hep (Figure 2.2). The reduction in HPSE-1 protein level was consistent over multiple experiments. The inability to completely inhibit HPSE-1 protein expression was possibly due to slow protein turnover rate. However, the reduction results in significant biologic effects in *in vitro* invasion assays or in *in vivo* tumorigenic assays, despite this small change in the protein level.

HPSE-1 activity was inhibited in a dose-dependent manner when cells were coinfected with Ad-S/hep and different doses of Ad-AS/hep (Table 2.2). Furthermore, we detected an increase in HPSE-1 activity with Ad-S/hep infection (Figure 2.3, Table 2.1). There was a small reduction of activity with Ad-AS/hep treatment compared to mock-treated cells (Figure 2.3A), which significantly decreased when HPLC retention times were calculated from these experiments ($P = .02$) (Figure 2.3B).

We were also able to inhibit the increased activity of Ad-S/hep with suramin, a known HPSE-1 inhibitor, therefore relating this augmented cell invasiveness to HPSE-1 (Figure 2.4). The poor sensitivity of HPSE-1 activity assays may reflect a lack of sensitivity at low HPSE-1 levels. Alternatively, differences between HPSE-1 activity and biologic assays can be due to lysis of the cells (HPSE-1 activity assays) versus using intact cells in *in vitro* (invasion assays) and *in vivo* (tumorigenic assays) analyses. In fact, we observed both a significant biologic effect in chemoinvasion assays and a reduction in both B16B15b and 70W cell-invasive properties (Figure 2.5). Of equal importance, mice injected with pAd5-Blue– and Ad-S/hep–treated 70W cells showed extensive tumor formation, whereas none of the mice injected with Ad-AS/hep–treated 70W cells presented any evidence of macroscopic malignancy (Figure 2.6, Table 2.3). Because control cells already expressed high levels of HPSE-1, we were unable to detect a significant difference between control and Ad-S/hep groups; however, this was found between the control and Ad-AS/hep groups ($P = .04$). Secondly, immunohistochemical analyses on the animal tissue from each group revealed more consistent and intense HPSE-1 staining in Ad-S/hep–infected cells (Figure 2.7, C and D) compared to using pAd5-Blue (Figure 2.7, A and B) or Ad-AS/hep vectors (Figure 2.7, E and F). These findings indicate that even a small reduction in HPSE-1 activity can lead to a great difference in its biologic function and may also suggest that the antisense-mediated gene delivery strategy can be a very effective way in preventing tumor formation and its metastatic potential.

Many chemotherapeutic agents are available to target HPSE-1 to suppress metastasis (Miao, Elkin et al. 1999; Parish, Freeman et al. 1999; Dhar, Gullbo et al. 2000; Meyers, Gagliardi et al. 2000; Edovitsky, Elkin et al. 2004). However, these agents have their limitations in affecting malignant cells as well as normal cells, thus affecting the normal growth and

functions of vital organs. Secondly, delivering these agents to specific sites can be difficult. Finally, interactions between the antimetastatic agents and biologic molecules like HSPG can interfere with normal biologic processes. Accordingly, to specifically inhibit the metastatic potential, which is a frequent malignant phenotype of many tumor types, a more specific therapeutic approach, such as the antisense-mediated RNA expression, needs to be considered. Antisense RNA can bind to the mRNA expressing the gene of interest and can block protein expression by the target cells.

Several groups have reported that tumor cells transfected with HPSE-1 cDNA acquire a highly metastatic phenotype *in vivo* (Vlodavsky, Friedmann et al. 1999) (reviewed in Ref. (Vlodavsky and Goldshmidt 2001)). Conversely, inhibition of HPSE-1 by means of antisense strategy, antimetastatic drugs, or gene silencing has resulted in decreased invasiveness *in vitro* (Zhang, Fu et al. 2003) and *in vivo* (Uno, Fujiwara et al. 2001; Edovitsky, Elkin et al. 2004). However, in other antisense animal models, tumor cells treated with viral vectors were directly injected into the thoracic cavity (Uno, Fujiwara et al. 2001), which bypasses several steps in the metastatic process that includes adhesion to endothelium and invasion (Parish, Coombe et al. 1987; Nakajima, Irimura et al. 1988; Vlodavsky, Bar-Shavit et al. 1991; Hulett, Freeman et al. 1999; Kussie, Hulmes et al. 1999; Toyoshima and Nakajima 1999; Vlodavsky, Friedmann et al. 1999; Vlodavsky and Friedmann 2001; Zetser, Bashenko et al. 2003; Sasaki, Higashi et al. 2004). A recent report of HPSE-1 inhibition by siRNA strategy also demonstrated reduced tumor load and increased survival in animals (Edovitsky, Elkin et al. 2004). Consistent with these findings, we have observed a significant inhibition of tumor formation in *in vivo* experiments where HPSE-1 – expressing 70W cells were infected with Ad-AS/hep and subsequently injected in athymic nu/nu mice (Figure 2.6, Table 2.3). This suggests that Ad-

AS/hep has the potential to suppress the metastatic phenotype of highly invasive tumor cells. Thus, approaches that selectively block the expression of molecules implicated in cellular invasion may be clinically more efficacious in preventing tumor cell dissemination than the ones used to induce tumor cell apoptosis.

The metastatic phenotype is considered decisive for tumor progression and several key molecules involved in these complex biologic events are potential candidates for therapeutic intervention. Our findings suggest that HPSE-1 is an attractive target for therapy and that its expression can be modified by adenoviral vectors to achieve favorable outcome in cancer metastasis.

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CHAPTER III
OVEREXPRESSION OF HEPARANASE INHIBITS BRAIN-METASTASIS *IN VIVO*

INTRODUCTION

Heparanase (HPSE) is an endo-β-D-glucuronidase that cleaves the heparan sulfate chains (HS) on heparan sulfate proteoglycans (HSPG) and thus contributes in extracellular matrix (ECM) degradation and remodeling. Conventionally, HPSE activity is associated with the metastatic potential of a variety of tumor-derived cell types (Vlodavsky and Friedmann 2001). HPSE protein expression was found to be up-regulated in various primary human tumors compared to surrounding non-cancerous tissues including hepatocellular (El-Assal, Yamanoi et al. 2001) and gastric carcinomas (Tang, Nakamura et al. 2002), bladder (Gohji, Okamoto et al. 2001), breast (Maxhimer, Quiros et al. 2002), and colon (Friedmann, Vlodavsky et al. 2000) cancers, pancreatic adenocarcinoma (Koliopanos, Friess et al. 2001; Kim, Xu et al. 2002; Rohloff, Zinke et al. 2002) and malignant melanoma (Murry, Greiter-Wilke et al. 2005). Over-expression of HPSE in lymphoma (Goldshmidt, Zcharia et al. 2002) or melanoma cells (Roy, Reiland et al. 2005) has been shown to enhance tumor metastasis and angiogenesis. Similarly, inhibition of HPSE expression with antisense or siRNA resulted in a significant decrease in melanoma metastatic phenotype (Edovitsky, Elkin et al. 2004; Roy, Reiland et al. 2005).

HPSE also facilitates cell invasion associated with autoimmune diseases, inflammation, and angiogenesis (El-Assal, Yamanoi et al. 2001; Elkin, Ilan et al. 2001; Gohji, Okamoto et al. 2001; Goldshmidt, Zcharia et al. 2002), possibly by releasing HS-bound angiogenic growth factors thereby activating these molecules. Accumulating evidence, including available clinical data, suggests that HPSE is an important molecular determinant in the progression of various primary and metastatic human tumors. However, an effect of HPSE overexpression or down

regulation in metastatic brain tumor has not been investigated so far. Based on the available basic research and clinical data, we hypothesized that HPSE overexpression in brain-metastatic melanoma would accelerate brain-tumor development, and inhibition of HPSE would have the opposite effect.

To explore this hypothesis, we used adenoviral vectors expressing the full-length human *hpse* gene in the sense (Ad-S/hep) or the antisense (Ad-AS/hep) orientations to specifically upregulate or inhibit HPSE protein expression. We were able to upregulate HPSE protein expression, activity, and *in vitro* invasive properties of the brain-metastatic melanoma cells (70W) by infecting the cells with Ad-S/hep and the opposite effects were observed with Ad-AS/hep in our previous report (Roy, Reiland et al. 2005). We were also able to demonstrate an inhibition of lung-metastasis formation by infecting the cells with the Ad-AS/hep *in vivo* whereas there was no significant difference in lung metastasis formation in the animals that were injected with cells that were either infected with the control (pAd5-Blue) or the Ad-S/hep vectors (Roy, Reiland et al. 2005).

However, in the previous study, we did not observe any brain-metastasis. Therefore, in the present report, we repeated the animal experiment, and to bypass the lungs, we injected the cells (70W) following various viral vector treatments, into the internal carotid artery of athymic nude (*nu/nu*) mice. As expected, at the end of the *in vivo* experiment, there was a complete inhibition of brain-tumor formation in all animals in the Ad-AS/hep group. We observed brain-tumor formation in both the control and the Ad-S/hep groups. Unexpectedly, animals in the Ad-S/hep group developed smaller and fewer tumors than the control group. Furthermore, some of the animals in the Ad-S/hep group did not have any brain tumors. We confirmed that the Ad-

S/hep was indeed overexpressing HPSE by using a HPSE activity assay with the pooled tumor tissue from animals.

MATERIALS AND METHODS

Materials

Heparan sulfate (HS) from bovine kidney was acquired from Sigma Chemical Company (St. Louis, MO). DMEM and Ham's F-12 nutrient medium and trypsin-EDTA were purchased from Gibco (Grand Island, New York, NY), and FBS from Hyclone Laboratories (Logan, UT). All other chemicals used were reagent grade or better.

Cells and Tissue Culture Conditions

Early-passage melanoma cells with metastatic abilities of human origin (70W line) (Ishikawa, Dennis et al. 1988; Ishikawa, Fernandez et al. 1988) were maintained as monolayer cultures in a 1:1 (v/v) mixture of DMEM/F-12 supplemented with 10% (v/v) FBS. Cells were maintained at 37°C in a humidified 5% CO_2/95% air (v/v) atmosphere and passaged using trypsin-EDTA (70W) before reaching confluence. The transformed embryonic kidney cell line 293 was grown in DMEM/F-12, supplemented with 10% FBS and penicillin (100 units/ml), and streptomycin (100 μg/ml). The 293 cells were used for the production of adenoviral vectors (Roy, Reiland et al. 2005).

Construction of Recombinant Adenovirus Containing the Human *Heparanase* Gene

An adenovirus expression vector kit (Takara Biomedicals Inc., Tokyo, Japan) was used to generate recombinant adenovirus for the expression of human HPSE gene in both sense and antisense orientations. Replication-deficient, E1- and E3-deleted recombinant adenovirus serotype 5 (Ad5) was used as the viral backbone. Plasmid DNA containing the cloned *hpse* gene and synthetic oligonucleotides Hep-5' (complementary to the 5' end of the gene) and Hep-

3' (complementary to the 3' end of the gene) were used to produce PCR products encompassing the *hpse* sequence (1632 bp). PCR products were treated with T4 DNA polymerase to generate uniformed blunt ends required for the ligation reaction into pAxCAwt cosmid provided by the Takara kit. After cloning the *hpse* gene into cosmid vector, availability of the recombinant cosmids containing the target gene in sense (5' - 3' under the CAG promoter of the vector) and antisense (3' - 5') orientations were confirmed by restriction analysis. Cosmid DNAs were produced in large quantities and after gradient purification were used for cotransfection with adenovirus genomic DNA-terminal protein complex (DNA-TPC provided by the kit) into 293 cells. Following the kit protocol, recombinant adenoviruses expressing human *hpse* in both sense (Ad-S/hep) and antisense (Ad-AS/hep) orientations were generated. Integrity of these recombinant viruses was confirmed by PCR and restriction analysis. The E1-, E3-deleted, replication deficient adenovirus pAd5-Blue was used as the control vector. Viral titer was quantified by determination of the 50% infectivity on tissue culture (TCID 50) in 293 cells (Roy, Reiland et al. 2005).

Adenoviral Infection

70W cells were plated on 100 mm dishes (10^6 cells/dish). Twenty four hours later, cells were washed twice with PBS containing 2 mM EDTA, and infected with virus diluted in serum-free DMEM/F-12. To maximize cell viability and protein expression, cells were infected with the viral vectors at a multiplicity of infection (M.O.I.) of 50. Plates were then incubated at 37°C for 1 hr, rocking gently every 10 min. Infection was terminated by adding culture medium containing penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were then incubated at 37°C for an appropriate period of time. Immunofluorescent control experiments were performed to confirm cell infectivity of the adenoviral vectors (Roy, Reiland et al. 2005).

Heparanase Activity Assays

HPSE activity was determined by degradation of FITC-HS using high-speed gel permeation column chromatography (HPLC) as previously described (Toyoshima and Nakajima 1999; Roy, Reiland et al. 2005). Briefly, homogenized tumor tissues (100 and 200 µg lysate/µg of HS) were prepared and incubated with FITC-HS at 37^{o}C for 18 hr in 100 µl of sodium acetate (100 mM, pH 4.2). Reaction was terminated by heating samples for 100^{o}C for 15 min. HS products yielded by HPSE reaction were analyzed by size exclusion chromatography performed on a HP1090 liquid chromatograph with a photodiode array detector (Agilent Technologies Inc., Wilmington, DE) and a HP1046 programmable fluorescence detector (Agilent Technologies Inc., Wilmington, DE). Samples (25 µl) were injected on a TSK-GEL 3000SW$_{XL}$ column (5 µm, 7.8 mm x 30 cm) with a TSK-GEL SW$_{XL}$ guard column (5 µm, 6.0 mm x 4.0 cm) from Tosoh Bioscience (Montgomeryville, PA) at an ambient temperature (25^{o}C). Samples were isocratically eluted using Tris-HCl (25 mM), NaCl (150 mM), pH 7.5 at a flow rate of 0.5 ml/min with a sample splitter placed between the column and detectors to maintain a column pressure of less than 70 bar. Fluorescence was monitored with excitation at 490 nm and emission at 520 nm, then data was processed and peaks were integrated using HP ChemStation software (Agilent Technologies Inc., Wilmington, DE). HPSE activity was determined by measuring the decrease in fluorescence intensity in the first one-half area of the intact FITC-HS peak chromatogram. The retention times were calculated from the highest peak of the chromatograms.

***In Vivo* Tumorigenic Assays**

Human malignant melanoma 70W cells were infected with Ad-S/hep, Ad-AS/hep and control pAd5-Blue at a M.O.I. of 50. Cells were harvested 24 hr after infection, and resuspended

in DMEM/F-12 at a density of 2.5 x 10^6 cells/ml. Female athymic *nu/nu* mice six-weeks old (n= 10 per group) were injected with 200 µl of cell suspension (5 x 10^5 cells) through a 31-gauge needle in the internal carotid artery (Schackert and Fidler 1988). Briefly, the mice were anesthetized with isoflurane, placed on a dissecting table with a heating pad on their backs, and examined under a dissecting microscope. The hair over the trachea was shaved and the neck was prepared for surgery with alcohol-iodine. The skin was cut by a median incision. After dissection, the trachea was exposed. The muscles were separated to expose the carotid artery, which was then separated from the vagus nerve. The artery was prepared for injection at a point proximal to the point of the division into the internal and external carotid arteries. A clamp was placed proximal to the site of injection and the cells (70W) were injected slowly with a 31 gauge needle. The injection site was glued with superglue and the clamp was removed. The skin flaps were apposed and stapled. At the end of two months, mice were euthanized and examined for tumor formation as previously described (Ishikawa, Fernandez et al. 1988). Metastatic behavior per animal group (number of brain tumor nodules and brains wet weight) was then determined.

RESULTS

Inhibition of Brain-Tumor Formation in Ad-S/hep - and Ad-AS/hep - Treated 70W Cells

To evaluate HPSE mechanism in metastatic cell invasion, human malignant melanoma 70W cells were infected with Ad-S/hep, Ad-AS/hep or pAd5-Blue vectors and injected in 6-weeks old female athymic nude (*nu/nu*) mice. Mice injected with pAd5-Blue and Ad-S/hep - treated 70W cells developed tumors but animals in the Ad-S/hep - treated cells had smaller and fewer tumor formation (Figure 3.1, Table 3.1). In addition, while all the animals in the pAd5-Blue group developed tumors (10/10), only 66% of the animals in the Ad-S/hep had tumors (6/9, we lost one animal following the procedure). As with our previous report, Ad-AS/hep inhibited

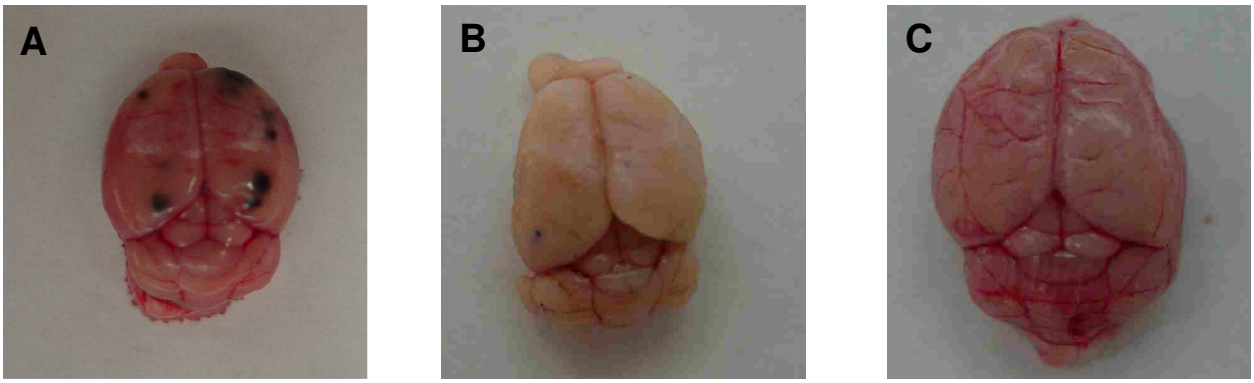


Figure 3.1. Overexpression of HPSE inhibits brain-tumor formation in athymic nude (*nu/nu*) mice. Human malignant melanoma 70W cells were infected with pAd5-Blue control vector (A), Ad-S/hep (B), or Ad-AS/hep (C) and were injected intracarotid in 6-week – old female athymic nu/nu mice. At the end of the experimental period, mice were euthanized and examined for tumor formation (black spots). Quantification of brain tumor formation and statistical analyses were performed on the number of experimental animals and are shown in Table 3.1.

Table 3.1 - Overexpression of HPSE inhibits tumor formation in nude (*nu/nu*) mice

Treatment	Number of Brain Tumor Nodules		Brain Weight (mg, mean ± SD)
	Median	Range	
pAd5-Blue	6	1-14[a]	422 ± 13
Ad-S/hep	2	0-8[b]*	432 ± 26
Ad-AS/hep	0	0[c]**	430 ± 16

a: 100% of mice had brain tumors. *b:* 66% of mice had brain tumors; *c:* none had brain tumors (*: $p < 0.01$, **: $p < 0.0003$ compared to control). Student's t test was used as statistical method.

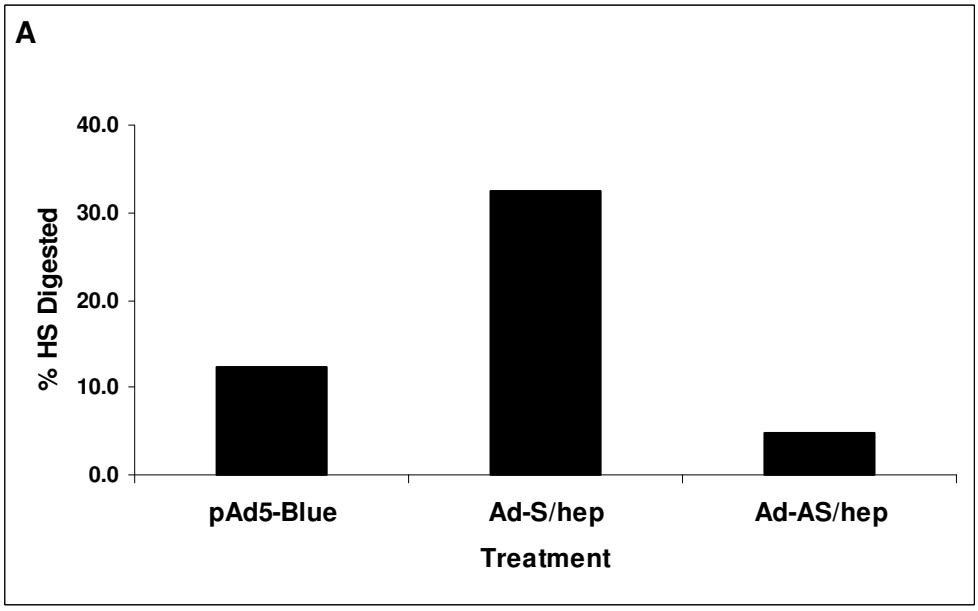
tumor formation in all of the mice treated with Ad-AS/hep (0/10) suggesting that the antisense adenoviral construct selectively inhibited HPSE-induced metastatic properties in 70W cells.

Heparanase Activity is Upregulated in Ad-S/hep–Treated Brain Tumor Tissue

To confirm that the Ad-S/hep was indeed overexpressing HPSE in the brain-tumors, we next performed the HPSE activity assay with pooled brain and tumor tissue from animals from each group. We used a specific HPSE activity assay on fluorescein isothiocyanate (FITC)-labeled HS in 70W cells. This assay detects HPLC profile shifts based on the size of HS fragments (Toyoshima and Nakajima 1999; Reiland, Sanderson et al. 2004; Roy, Reiland et al. 2005). Since HPSE cleaves the HS chains into discrete fragments, we detected a shift in the profile when compared to FITC-HS alone which was used as negative control (Toyoshima and Nakajima 1999; Reiland, Sanderson et al. 2004; Roy, Reiland et al. 2005) (data not shown). We observed a decrease in fragment size in Ad-S/hep-treated tumor tissue compared to pAd5-Blue treatment, which indicated increased HPSE activity in the Ad-S/hep group (Figure. 3.2). HPSE activity was increased by three fold in the Ad-S/hep group compared to pAd5-Blue by calculating percent of HS digestion (Figures 3.2A and B). We also observed HPSE activity in the brain tissue of Ad-AS/hep treatment group.

A prominent difference in the HPLC retention times was seen between the Ad-S/hep and the control. These retention times reflect differences in the size of the predominant HS fragments (Toyoshima and Nakajima 1999; Reiland, Sanderson et al. 2004; Roy, Reiland et al. 2005). The brain-tumor tissue in the Ad-S/hep group showed a higher retention time compared to Ad-AS/hep and pAd5-Blue treatment groups suggesting that increased digestion of HS chains resulted from an enhanced HPSE activity (Figures 3.2C and D).

Figure 3.2. HPSE activity is upregulated in brain tumor tissue of animals in Ad-S/hep treatment group compared to pAd5-Blue. Tumor tissue with surrounding normal brain tissue (pAd5-Blue and Ad-S/hep), or normal brain (Ad-AS/ hep) were analyzed for HPSE activity. Equal amounts [100µg (A and C) and 200 µg (B and D)] of tissue were incubated with FITC-HS for 18 hours (see Experimental Procedures section) and run on HPLC to demonstrate HPSE activity. (A and B) Percentage of HS digested with different treatments. (C and D) Retention times calculated with different treatments.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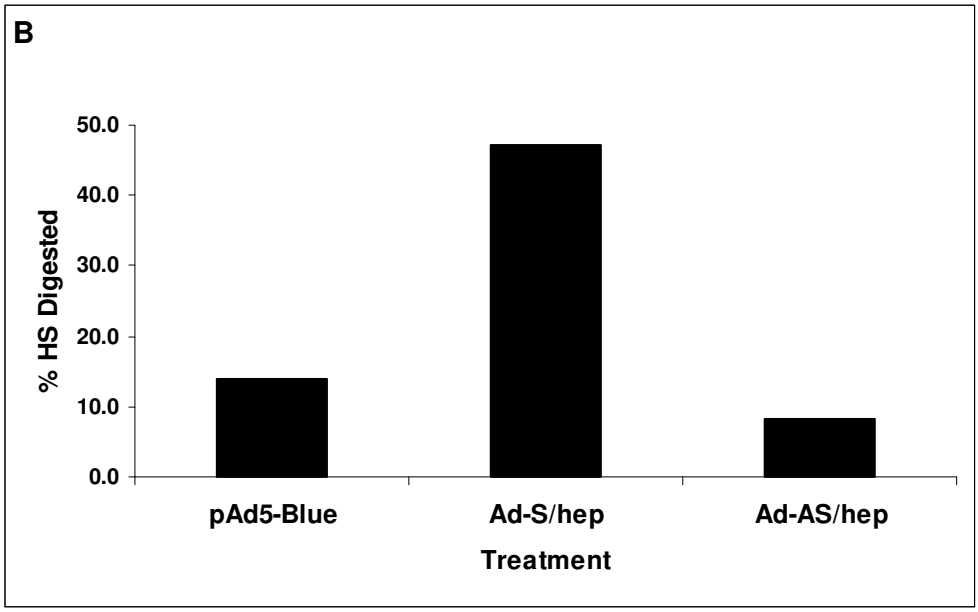
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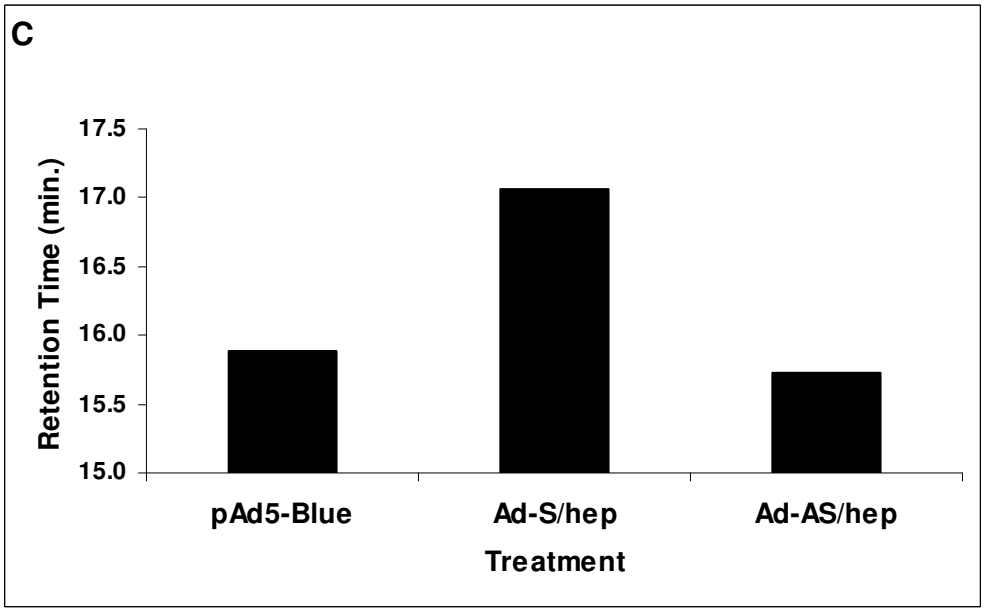
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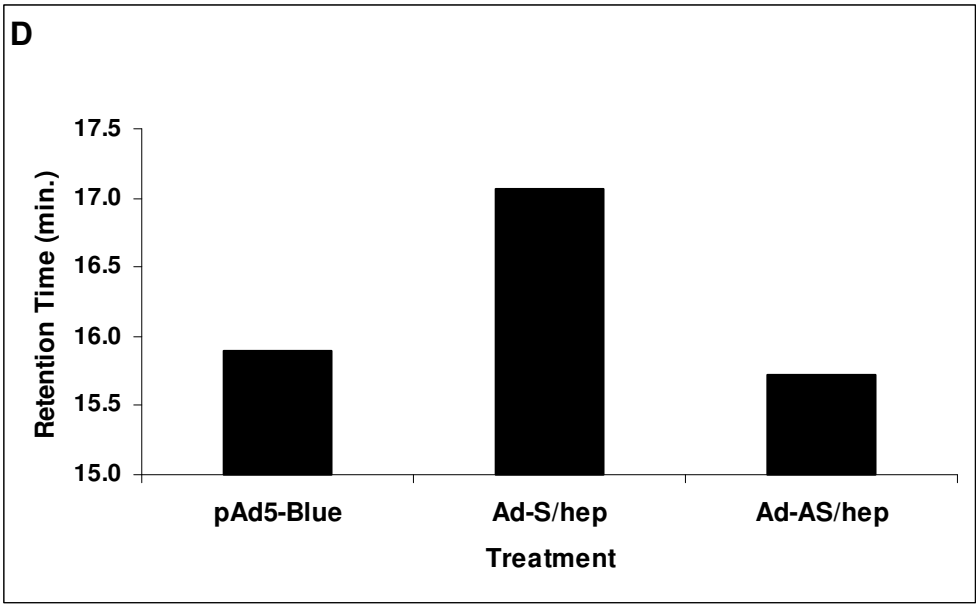
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DISCUSSION

Over the past two decades, HPSE activity has been associated with the metastatic potential of various tumor derived cell lines and in both primary and secondary tumor development. Prometastatic properties of HPSE are attributed mainly to its involvement in ECM remodeling and liberation of various HS-bound growth factors (Nakajima, Irimura et al. 1983; Vlodavsky, Fuks et al. 1983; Nakajima, Irimura et al. 1986; Ricoveri and Cappelletti 1986; Nakajima, Irimura et al. 1988). The cloning of a single human *heparanase* cDNA sequence reported by several groups independently (Hulett, Freeman et al. 1999; Kussie, Hulmes et al. 1999; Toyoshima and Nakajima 1999; Vlodavsky, Friedmann et al. 1999) suggested that mammalian cells express only one dominant HS-degrading enzyme. This is in contrast to the large number of proteases that are capable of degrading and remodeling the variety of polypeptides present in the ECM (Werb 1997; Sanderson R.D. 2005) and emphasizes the biological importance of this glycosidase. A number of studies have now presented evidence that HPSE overexpression in tumor cells enhances tumor growth and angiogenesis (Uno, Fujiwara et al. 2001; Vlodavsky, Goldshmidt et al. 2002; Sanderson, Yang et al. 2004; Roy, Reiland et al. 2005).

Surprisingly, in our study using the brain-metastatic melanoma cells, overexpression of HPSE led to inhibition of tumor formation. We confirmed that the Ad-S/hep was indeed overexpressing HPSE in the brain-tumors with a HPSE activity assay. HPSE activity was increased by three fold in the Ad-S/hep group compared to pAd5-Blue by calculating percent of HS digestion (Figures 3.2A and B). We also observed HPSE activity in the brain tissue of Ad-AS/hep treatment group. The low levels of HPSE activity in the Ad-AS/hep group was possibly due to HPSE expressed by the endothelial cells and the astrocytes (Marchetti 1997; Marchetti, Li et al. 2000), since there were no detectable tumors in the brains of these animals (Figures 3.2A

and B). This is in agreement with the recent report by Zetser et al. (2003) using a glioma model. In that report, moderate levels of HPSE stimulated tumor growth and invasion, whereas higher levels produced the opposite effect and inhibited tumor development, indicating a concentration-dependence for its activity (Zetser, Bashenko et al. 2003).

This unexpected finding of inhibition of tumor formation led us to hypothesize that extensive remodeling of the ECM and cell-surface HS by higher concentrations of HPSE disrupted the growth factor-mediated activity in the brain-metastatic melanoma cells, since much of the pro-metastatic and the pro-angiogenic activities of HPSE are primarily due to modification of the HS-bound growth factor activities (Reiland, Kempf et al. 2006). This possibility has been investigated by treating the human brain-metastatic melanoma (70W) cells with various concentrations of HPSE and exploring its effect on fibroblast growth factor-2 (FGF2)-mediated signaling and activity (Reiland, Kempf et al. 2006). FGF2 was chosen because it is an important mediator of melanoma angiogenesis and progression (Herlyn 2005). Furthermore, HS sequence and length affect FGF2 activity, and binding to HS by both FGF2 and FGF receptors (FGFR) is essential for its signaling and subsequent pro tumorigenic activities (Rapraeger 1995). HPSE at low concentrations enhanced FGF2 binding and FGF2-mediated signaling and angiogenesis, whereas the opposite effects were observed with supraphysiological concentrations of HPSE (Reiland, Kempf et al. 2006). That study demonstrating HPSE's ability to both augment and inhibit FGF2 binding, signaling and FGF2-mediated angiogenesis in a concentration-dependent manner could explain the unexpected tumor inhibitory phenomenon by high levels of HPSE (Reiland, Kempf et al. 2006).

Extensive remodeling of cell surface HS may hinder growth factor binding and internalization (Iozzo 2001; Sanderson 2001). However, a modest degradation of cell surface

HS by low levels of HPSE, which more closely resembles *in vivo* expression levels, possibly alters the HS structure resulting in either decreased or increased interactions with different growth factors. Furthermore, HPSE degradation of HS could change the availability of HS-bound growth factors in the tumor microenvironment by liberating them from the ECM and the cell surface.

In the report by Zetser et al. (2003) in the glioma model, the inhibitory effect of high HPSE expression on tumor growth was due to a decrease in cell proliferation. HPSE also increased adhesive properties in the cells at both low and high concentrations by inducing β-1 integrin expression which is associated with aggressive properties of tumor cells. According to their study, while adhesion to vascular endothelial cells and subendothelial matrix was promoted by low levels of HPSE, a further increase in HPSE expression led to spreading of cells in tight monolayer that hindered metastasis formation (Zetser, Bashenko et al. 2003).

Our work in brain-metastatic melanoma cells not only extends the concept that HPSE alters tumor responsiveness in multiple systems, but also suggests that these changes are due to HPSE-mediated HS remodeling of the tumor cell surface and the ECM (Reiland, Kempf et al. 2006). Interestingly, this inhibitory effect by high levels of HPSE has only been reported in the brain tumor models, indicating the possibility that there is a more complex regulation of this enzyme in the brain.

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CHAPTER IV
HEPARANASE-DEGRADED CELL-SURFACE HEPARAN SULFATE PROMOTES MELANOMA CELL MIGRATION AND ANGIOGENESIS

INTRODUCTION

Enzymatic remodeling of heparan sulfate proteoglycans (HSPG) within the tumor microenvironment is emerging as an important mechanism for dynamic regulation of tumor growth (Sanderson, Yang et al. 2004; Sanderson 2005). HSPG are essential and integral components of the cell surface and extracellular matrix (ECM) that surrounds all mammalian cells. This is a family of glycoproteins that is distinguished by the covalent attachment of one or more heparan sulfate glycosaminoglycan chains (HSGAG) to their protein core (Kjellen and Lindahl 1991). In addition to providing structural integrity, they act as a storage depot for a variety of heparan sulfate (HS)-binding proteins, including growth factors and chemokines (Kjellen and Lindahl 1991). HSGAG 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 normal host cells, HSGAG mediate tumor and host cell interactions (Raman, Venkataraman et al. 2003). HSGAG also influence tumor metastasis to sites such as the brain by arbitrating interactions between cancer cells, platelets, endothelial cells, and host organ cells (Varki and Varki 2002). Intact HSGAG prevent metastasis by acting as a physical barrier in the ECM. Heparanase (HPSE) secreted by tumor cells degrades HSGAG on the cell-surface and ECM, and thereby decreases the integrity of the barrier to malignant cell invasion (Vlodavsky and Friedmann 2001; Sanderson 2005).

HPSE is an endo-β-D-glucuronidase that cleaves HS at specific intrachain sites, resulting in fragments of appreciable size (10–20 sugar units) (Nakajima, Irimura et al. 1984; Nakajima, Irimura et al. 1988; Marchetti 1997; Vlodavsky, Goldshmidt et al. 2001). Various *in vitro* and *in*

vivo studies have suggested a role for HPSE in tumor invasion and metastasis (Hulett, Freeman et al. 1999; Vlodavsky, Friedmann et al. 1999; Koliopanos, Friess et al. 2001; Uno, Fujiwara et al. 2001; Goldshmidt, Zcharia et al. 2002; Kelly, Miao et al. 2003; Zetser, Bashenko et al. 2003; Roy, Reiland et al. 2005). In addition, HPSE activity is upregulated in human cancers; namely, a number of descriptive studies on clinical samples demonstrated a correlation between tumor malignancy and HPSE levels (Nakajima, Irimura et al. 1988; Friedmann, Vlodavsky et al. 2000; Vlodavsky, Elkin et al. 2000; Koliopanos, Friess et al. 2001; Uno, Fujiwara et al. 2001; Kim, Xu et al. 2002; Maxhimer, Quiros et al. 2002; Tang, Nakamura et al. 2002; Kelly, Miao et al. 2003; Doweck, Kaplan-Cohen et al. 2006).

In vivo animal studies also indicate 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 (Liu, Shriver et al. 2002a; Dai, Yang et al. 2005; Narita, Staub et al. 2006; Reiland, Kempf et al. 2006; Xu, Rao et al. 2007). Depending on HS composition or sequences that are involved in the process of tumorigenesis, it can either act as pro-tumorigenic, or anti-tumorigenic agents (Liu, Shriver et al. 2002a; Reiland, Kempf et al. 2006). Soluble HS or HS in the ECM can also differentially regulate FGF-2 binding and signaling, leading to modification of angiogenesis of brain-metastatic melanoma cells depending on the HS concentration (Reiland, Kempf et al. 2006).

Some of the HS-binding growth factors that are important for angiogenesis and tumor development are fibroblast growth factors (FGF1 and FGF2), vascular endothelial growth factor (VEGF), hepatocyte growth factor, transforming growth factor-β, and platelet-derived growth factor (Nurcombe, Smart et al. 2000; Sasisekharan and Venkataraman 2000; Iozzo 2001; Sasisekharan, Shriver et al. 2002; Sasaki, Higashi et al. 2004). VEGF (Senger, Galli et al. 1983;

Senger, Perruzzi et al. 1986) was initially described as a highly specific mitogen for endothelial cells (Leung, Cachianes et al. 1989) and as a potent inducing agent for angiogenesis and vasculogenesis in a variety of physiological and pathological conditions (Plate, Breier et al. 1992; Ferrara, Winer et al. 1993; Tuder, Flook et al. 1995; Ferrara and Davis-Smyth 1997). Inhibition of VEGF signaling by VEGF antagonists, antisense VEGF, or dominant negative VEGFR (VEGF receptor) impaired tumorigenesis *in vivo* (Kim, Li et al. 1993; Millauer, Shawver et al. 1994; Saleh, Stacker et al. 1996; Yano, Shinohara et al. 2000). The VEGF family of proteins includes VEGF A through E and placenta growth factor (Neufeld, Cohen et al. 1996; Ranieri, Patruno et al. 2006). VEGF A is the most studied and one of its splice variants, VEGF165, is the most potent and widely expressed isoform known (Plouet, Moro et al. 1997). VEGF165 is secreted as a disulfide-linked homodimer with two identical heparin-binding sites. HS, by binding to VEGF, regulates the diffusion, half-life, and affinity of VEGF165 for its cognate receptors (Gallagher 2001; Iozzo 2001).

We sought to assess the role of HPSE-degraded cell-surface HSGAG in VEGF165 signaling in brain-metastatic melanoma since VEGF is known to be essential for brain metastasis in melanoma (Yano, Shinohara 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 demonstrated that the isolated cell-surface HSGAG stimulated migration but not proliferation of melanoma cells (B16B15b) *in vitro*, and that HSGAG also promoted angiogenesis *in vivo,* as assessed by the Matrigel™ plug assay. Interestingly, the melanoma cell-surface HSGAG did not stimulate murine brain endothelioma cell migration (b.End3) *in vitro*. Finally, we attempted to characterize the isolated HS by ion-pair HPLC. Our results suggest that, in addition to remodeling the ECM and releasing growth

factors and chemokines, HPSE may contribute to the aggressive phenotypes of melanoma by releasing bioactive HS that might stimulate melanoma migration and angiogenesis independent of any growth factor or chemo/cytokine activity.

EXPERIMENTAL PROCEDURES

Materials

Heparan sulfate (HS) from bovine kidney and heparin lyase II (heparinase II, no EC) derived from *Flavobacterium heparinium* were purchased from Sigma Chemical Company (St. Louis, MO). Heparin-lyase I (heparinase, EC 4.2.2.7) and heparin lyase III (heparitinase, EC 4.2.2.8) from *Flavobacterium heparinium* were obtained from Seikagaku (Seikagaku America, Falmouth, MA). Heparin disaccharide standards were purchased from Sigma and Grampian Enzymes (Scotland, UK). DMEM and Ham's F-12 nutrient medium and trypsin-EDTA were purchased from Gibco (Grand Island, New York, NY), and FBS from Hyclone Laboratories (Logan, UT). Reduced-growth factor Matrigel^{TM} 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 melanoma cells with high metastatic abilities of murine (B16B15b line) (Marchetti 1997) origin were maintained as monolayer cultures in a 1:1 (v/v) mixture of Dulbecco's modified Eagle's medium/F-12 supplemented with 5% (v/v) fetal bovine serum. Murine brain endothelioma cells (b.End3) (Montesano, Pepper et al. 1990) were passaged in Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 10% 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 3 times in DMEM/F-12 containing 0.1% BSA, penicillin (100 U/ml) and streptomycin (100 µg/ml), then incubated with indicated concentrations (0-5 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 37ºC in a shaker incubator at 50 rpm.

HPSE Isolation and Activity

Recombinant human HPSE was purified as previously described (McKenzie, Young et al. 2003; Reiland, Kempf et al. 2006). Briefly, Sf9 insect cells, transfected with baculovirus transfer vectors containing HPSE subunits, were grown in SF900II serum-free medium (Gibco BRL, Grand Island, NY) for high-titer stocks. Tni insect 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, 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 Degrading Enzyme Assay Kit; Takara Mirus, Madison, WI) (Reiland, Sanderson et al. 2004). HPSE eluted at 0.67 M NaCl, as expected (McKenzie, Young et al. 2003; Reiland, Kempf et al. 2006).

Flow Cytometric Analyses

Degradation of cell-surface HS was confirmed by flow cytometric analyses. Briefly, B16B15b metastatic melanoma cells were treated with different concentrations of HPSE (0-5000 ng/ml) overnight or Hep III (10 µg/ml) for 1 hour at 37^{o}C on a shaker incubator. The medium was removed at the end of the treatment and the cells were released by PBS-EDTA. Cells (1 x

10^6) were incubated with HS mAb 3G10 (Seikagaku) 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% paraformaldehyde and stored at 4°C till analysis. Samples were analyzed for cell-surface HSGAG staining using FACScan Flowcytometer. Data were analyzed with WinMDI. Appropriate control samples were run to subtract background staining.

Isolation of HSGAG from Cell-Surface

B16B15b metastatic melanoma cells were treated with or without HPSE (5000 ng/ml) overnight at 37°C on a shaker incubator. Conditioned medium was collected and pH was adjusted to 6.0. The medium was then centrifuged at 5000 rpm for 10 minutes, filtered through 0.22µm filters and boiled for 10 minutes. The medium was incubated with DEAE-Sephacel (Sigma) overnight and subsequently poured onto columns. HSGAG fragments bound to DEAE were washed with 0.1 M sodium phosphate and 0.15 M NaCl, pH 6.0, in the column. The fragments were gradient eluted with 0.5 M, 1.0 M, 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, the isolated fragments were treated with pronase (Sigma) to remove protein contamination from isolated HSGAG.

HPSE-degraded HSGAG used in biological experiments were analyzed by separating HSGAG on a Criterion 4-20% TBE gel (Bio-Rad Laboratories, Hercules, CA) for 20 min at 60 mAmp. Bands were visualized with alcian blue 8GX (Sigma-Aldrich) followed by silver staining (Pierce Endogen, Rockford, IL) (Pervin, Gallo et al. 1995). Densitometric analyses were performed using a Versadoc imaging system (Bio-Rad Laboratories) to determine the

profile leading edge. Non-treated commercial HS from bovine kidney were simultaneously run at various concentrations to obtain quantitative analysis.

Western Blotting Analyses

For signal transduction studies, cells were plated in 12 or 24-well tissue culture wells for 24-48 h to allow cell adhesion. HPSE/Hep III treatments of cell surface HS were performed as described above. Cells were exposed to 0-100 ng/ml of each of FGF2 or VEGF or HS in triplicates for 10-30 min at 37°C. Where indicated, FGF2/VEGF were added together with 0-100 ng/ml HS. Following stimulation, the medium was aspirated from cells and 100-200 µl of lysis buffer [20 mM Tris-HCl (pH 7.4) containing 1% SDS, 1 mM PMSF, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.1 mM sodium molybdate, 1 mM sodium orthovanadate, and 5 µg/ml aprotinin] was added at 4ºC. Lysed cells were scraped from the dish and put through three to six cycles of freezing in liquid nitrogen and heating to 100°C for 5 min. Samples for Western blots were incubated at 100°C for 5 min with Laemmli sample buffer and separated on a 4-15% Criterion gel (Tris-HCl, Bio-Rad Laboratories). Proteins were transferred to PDVF membrane (Pierce Endogen). Membranes were incubated in a blocking reagent [5% (w/v) non-fat dry milk, 0.3% Tween-20 in BSA in TBS] for 2 h. Membranes were then incubated for 16 h in antibody dilution buffer (5% BSA, 0.1% Tween-20 in TBS) with the following primary antibodies: anti-phosphorylated-ERK (threonine 202/tyrosine 204) monoclonal antibody, anti-phosphorylated FAK (serine 910) polyclonal antibody, anti-phosphorylated AKT (serine 473) monoclonal antibody , anti-phosphorylated p38 monoclonal antibody (Cell Signaling Technology, Beverly, MA), anti-phosphorylated FAK (tyrosine 397) polyclonal antibody (Biosource International, Camarillo, CA) at dilutions of 1:1000, or Vinculin at a dilution of 1:2000 (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed

for 1 h with 6-8 changes of TBS containing 0.1% Tween-20 and incubated with horseradish peroxidase-anti-rabbit IgG (1:10,000 dilution; Accurate Chemical and Scientific Co., Westbury, NY). Membranes were then washed and developed using the Super-signal west femto maximum sensitivity substrate (Pierce Endogen). Labeling was detected and quantified using a Versadoc imaging system (Bio-Rad Laboratories).

Wound Healing Assays

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 3 times in DMEM/F-12 containing 0.1% BSA, penicillin (100 U/ml) and streptomycin (100 µg/ml) and then incubated with the same medium for an hour. Using a sterile 100 µl tip, a single scratch was made through the middle of each well. The medium was subsequently removed and the wells were rinsed three times with DMEM/F-12 containing 0.1% BSA and penicillin/streptomycin to remove the detached cells. Indicated concentrations of cell-surface HSGAG, or recombinant FGF2/VEGF were added to cells in triplicates in DMEM/F-12 containing 0.1% BSA, 4mM Hepes, penicillin (100 U/ml), and streptomycin (100 µg/ml) for 8 h at 37ºC in a humidified 5% CO_2/95% air (v/v) atmosphere. Photomicrographs were taken at 0 hour (T_0) and at the end of the experiment (T_8) using identical conditions to calculate percentage of relative gap closure. Relative gap closure was measured as $[1-(T_8/T_0)]$ for B16B15b. Migration assays for endothelioma were performed for 18 or 24 h, hence relative gap closure for endothelioma experiments were calculated as $[1-(T_{18 \text{ or } 24}/T_0)]$.

Proliferation Assays

Proliferation of melanoma cells was assayed by alamarBlue™ (BioSource International, Camarillo, CA, USA) which monitors the reducing environment of the proliferating cell as per

manufacturer's instructions. Briefly, 1 x 10^4 cells/ml were plated into 24 well plates in triplicates and incubated and allowed to attach for 24 h. At the start of the proliferation assay, cells were washed 3 times in DMEM/F-12 containing 0.1% BSA and penicillin (100 U/ml) and streptomycin (100 µg/ml), and then incubated with the same medium for an hour. Indicated concentrations of cell-surface HSGAG or recombinant VEGF were added to cells in DMEM/F-12 containing 0.1% BSA, 4 mM Hepes, penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37ºC in a humidified 5% CO2/95% air (v/v) atmosphere. For indicated time points alamarBlue™ (10% v/v) was added per well. Plates were incubated for 4 h at 37°C in a humidified 5% CO_2/95% air (v/v) atmosphere before spectrophotometric readings were taken. 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* Angiogenesis Assays**

B16B15b cells were released with PBS-EDTA, washed two times in DMEM/F-12, and resuspended at 1x10^7 cells/ml in 50% reduced-growth factor Matrigel™ (Becton Dickinson, Labware, Bedford, MA) in DME/F-12 at 4°C. HSGAG fragments and VEGF were added accordingly. Cells (2 x 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). Mice were sacrificed on the 10^{th} day. Tumors were excised, fixed in 10% formalin, and embedded in paraffin. Tumor sections (7 µm thick) were then H&E-stained and examined under the microscope. Blood vessel density was assessed by counting all blood vessels within the tumor region in five sections in each tumor. Tumor sections were photographed using an Olympus DP70 camera attached to an 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 using a stage micrometer 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.

Characterization of Cell-Surface HSGAG

Isolated B16B15b melanoma cell-surface HSGAG were digested into disaccharides in 20 mM acetate buffer, pH 7.0, containing 1 μmol of calcium acetate and 5 μg/ml of each of heparin lyases I, II and III at 37°C overnight. Digestions were terminated by boiling for 10 min, and following centrifugation in an Eppendorf microfuge (10,000 rpm) for 5 min, aliquots were taken for direct ion-pair HPLC analysis of variously sulphated Δ-disaccharides.

Separation of the variously sulphated Δ-disaccharides, isolated by HPSE-degradation from melanoma cell-surface followed by degradation with the various heparin- and HS lyases, was carried out by ion-pair reversed-phase chromatography (Karamanos, Vanky et al. 1997). This chromatography was performed on an Agilent 1100 system (Agilent Technologies Inc., Wilmington, DE), using a 4.6 x 150 mm, 5μm I.D. Agilent Zorbax Eclipse XDB-C18 column connected to a 7.5x4.6 Altech Altima C18 5 μM guard column. The flow-rate was 1.25 ml/min and the following elution program was used: isocratic elution with 100% A for 8 min, gradient

elution to 53.3% B over 26 min, gradient elution to 75% B over 30 min, followed by another gradient elution to 100% B over 35 min. The column was then washed and re-equilibrated by further elution with 100% A for 5 min (Karamanos, Vanky et al. 1997). The absorbance of the column elute was monitored at 232 nm (Karamanos, Vanky et al. 1997). The types of the various Δ-disaccharides produced by enzymatic actions of heparin- and HS lyases on HS were determined by injecting standard disaccharides, and their compositions were compared to the peak heights and/or peak areas obtained with those from external standard disaccharides (Karamanos, Vanky et al. 1997). An internal standard (IP) was used in all samples to control technical error (Saad, Ebel et al. 2005). The linearity of the detector response was tested using precisely known amounts of 10 heparin disaccharides (Table 4.1) (Karamanos, Vanky et al. 1997; Saad, Ebel et al. 2005). Data were normalized by calculating the ratio of peak area of standards to that of internal control IP.

RESULTS

Removal and Isolation of Cell-Surface HSGAG with HPSE Treatment

Highly brain-metastatic B16B15b melanoma cells were chosen as a source of HSGAG since they have high expression of HSGAG on the cell surface. The extent of HS degradation by HPSE was assessed by detection of cell-surface HS by FACS analysis. Detectable reduction in HSGAG levels was seen with as low as 5 ng/ml HPSE compared to no HPSE treatment (Fig. 4.1A). When cells were treated with higher HPSE concentrations (50-5000 ng/ml), a dose-dependent decrease in cell surface HSGAG was observed (Fig. 4.1A).

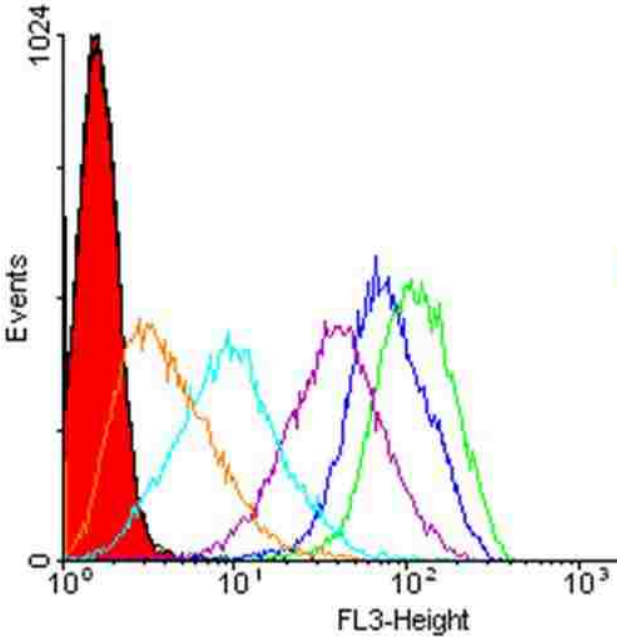
Conditioned medium following HPSE treatment (5μg/ml) from B16B15b melanoma cells was collected and HSGAG were isolated by ion-exchange column chromatography (Fig. 4.1B). Assessment of HPSE-mediated HSGAG degradation was determined by gel electrophoresis of

Table 4.1. Heparin disaccharide standards

Sugar	Nomenclature
ΔUA-2S→GlcNS-6S	I-S
ΔUA→GlcNS-6S	II-S
ΔUA-2S→GlcNS	III-S
ΔUA→GlcNS	IV-S
ΔUA-2S→GlcNAc-6S	I-A
ΔUA→GlcNAc-6S	II-A
ΔUA-2S→GlcNAc	III-A
ΔUA→GlcNAc	IV-A
ΔUA-2S→GlcNCOEt-6S	I-P (internal std)
ΔUA-2S→GlcN-6S	I-H
ΔUA→GlcN-6S	II-H
ΔUA-2S→GlcN	III-H
ΔUA→GlcN	IV-H

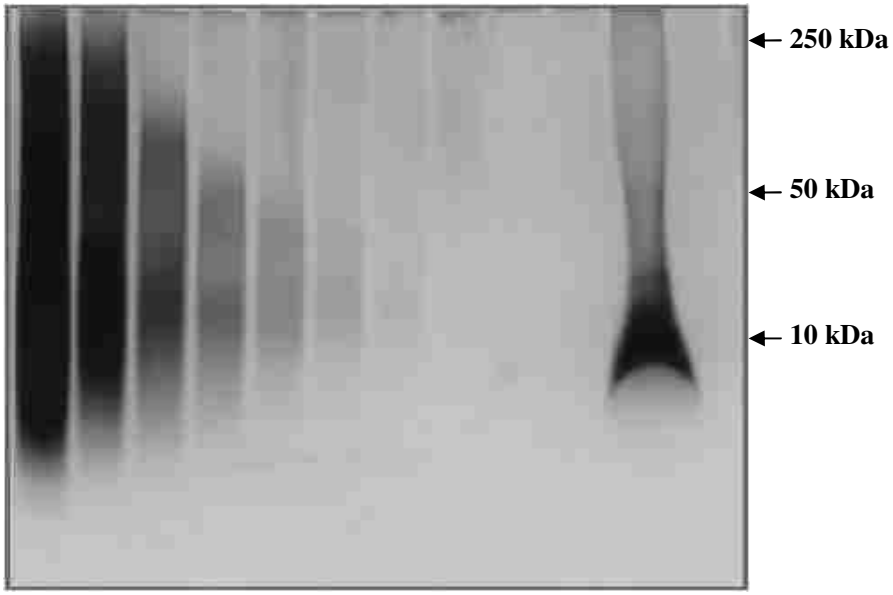
Figure 4.1. Dose-dependent reduction of cell surface HSGAG with HPSE. A. Murine B16B15b melanoma cells were treated with 0-5000 ng/ml HPSE. Cell-surface HS level were detected by flowcytometry. HPSE removes cell-surface HSGAG in a dose-dependent manner, (green) 0 ng/ml HPSE, (blue) 5 ng/ml HPSE, (purple) 50 ng/ml HPSE, (light blue) 500 ng/ml HPSE or with (orange) 5000 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, enzymatic digestions generate smaller fragment that migrate faster on SDS PAGE compared to untreated commercial HS. Various concentrations of untreated HS were run to generate a standard curve for determination of concentration of HSGAG after densitometric analysis. Gel is representative of at least three experiments.

A.



Relative Fluorescence Intensity (HS)

B.



HS conc. (ng/ml) 1000 333 111 37 12 4 0 HS (B16B15b)

isolated fragments. Since the isolated HSGAG is a heterogeneous mixture of oligosaccharides due to HPSE digestion, it migrates as a broad band during gel electrophoresis (Fig. 4.1B). HPSE-mediated HS degradation products migrate faster compared to untreated commercial HS indicating a reduction in HS fragment size as expected. The leading edge of HSGAG profiles was determined after densitometric analysis and concentration was determined by generating a curve with known standards (Fig. 4.1B).

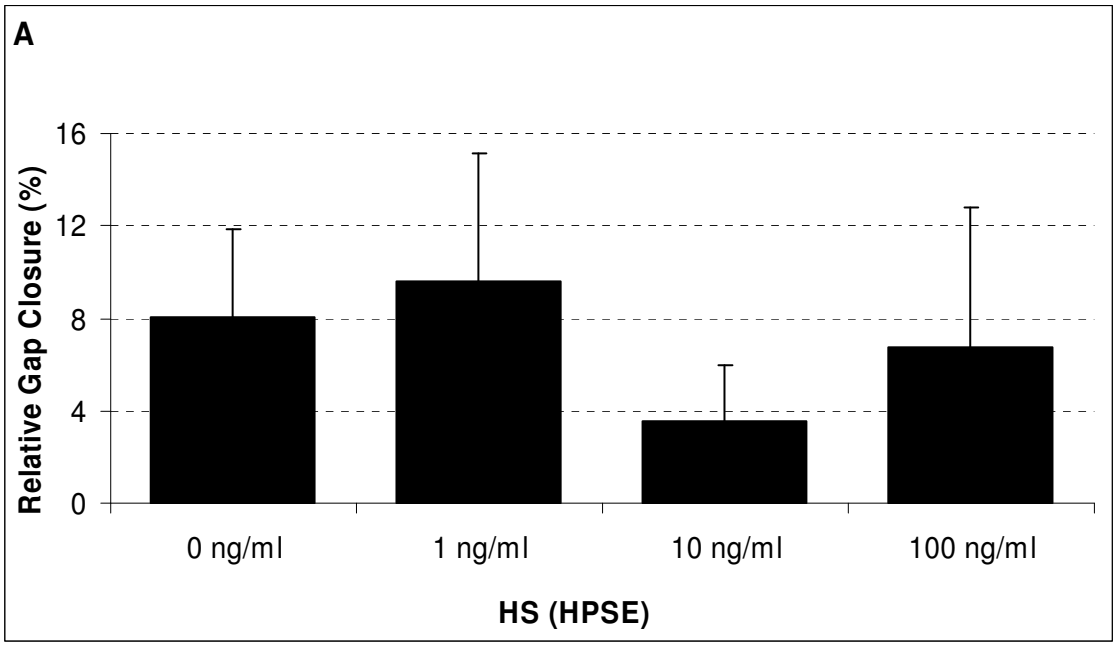
Effects of HPSE-Degraded HSGAG on Endothelioma *In Vitro*

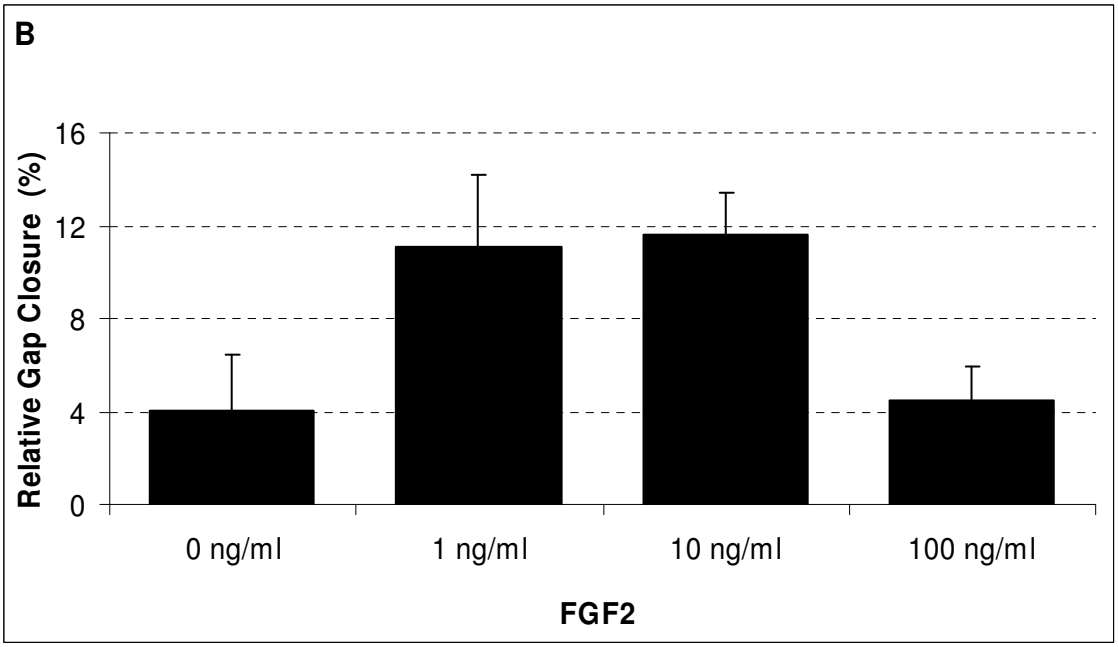
To study how exogenous addition of HS will influence endothelioma (b.End3) biological activity, we added HPSE-digested bovine kidney HS to serum-free endothelioma medium. HS at the concentration range of 0-100 ng/ml did not have any effect on migration (Fig. 4.2A). We also used FGF2, a known mitogenic factor for endothelial cells, at concentrations of 0-100 ng/ml (Fig. 4.2B). At low concentration (1 and 10 ng/ml), FGF2 stimulated migration compared to no FGF2 treatment, but at higher concentrations (100 ng/ml), it did not affect endothelioma cell migration (Fig. 4.2B).

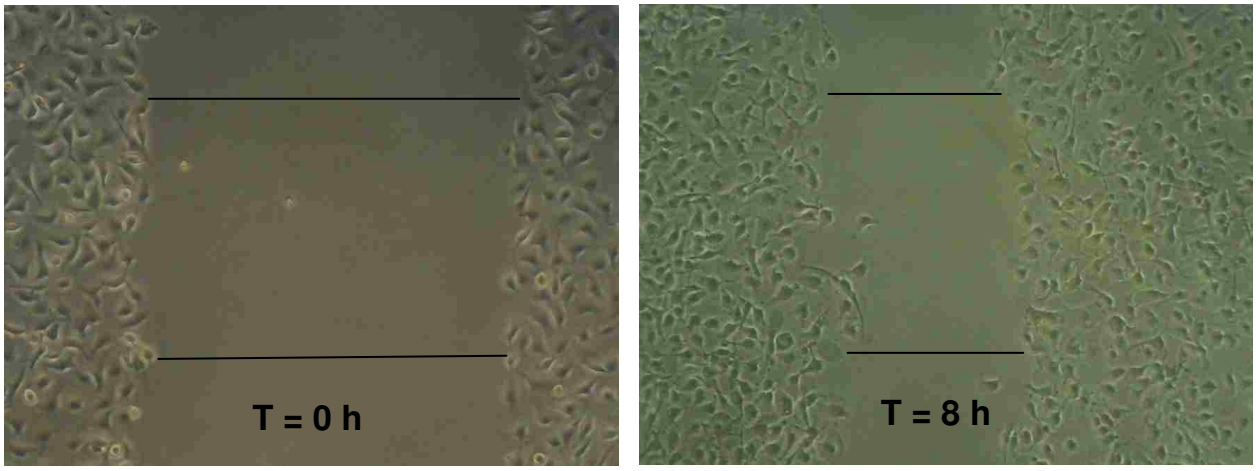
To study the underlying signaling mechanisms, we treated b.End3 cells with 1-100 ng/ml of FGF2 for 10-30 minutes and analyzed for phosphorylation of ERK (thr 202/tyr 204), AKT (ser 473), FAK (tyr 397 and ser 910), and p38 proteins since these molecules are important determinants of tumor survival. Similar to previous migration study, FGF2 at low concentration stimulated phosphorylation of ERK compared to no FGF2 treatment. At a higher concentration (100 ng/ml), FGF2 did not affect phosphorylation of ERK (Fig 4.3A). Phosphorylation of AKT, FAK or p38 were unaffected by FGF2 in endothelioma (Fig 4.3).

Next, we investigated effects of VEGF at similar concentrations on b.End3. Unlike FGF2 experiments, VEGF was able to stimulate phosphorylation of ERK at 1 ng/ml, but strong

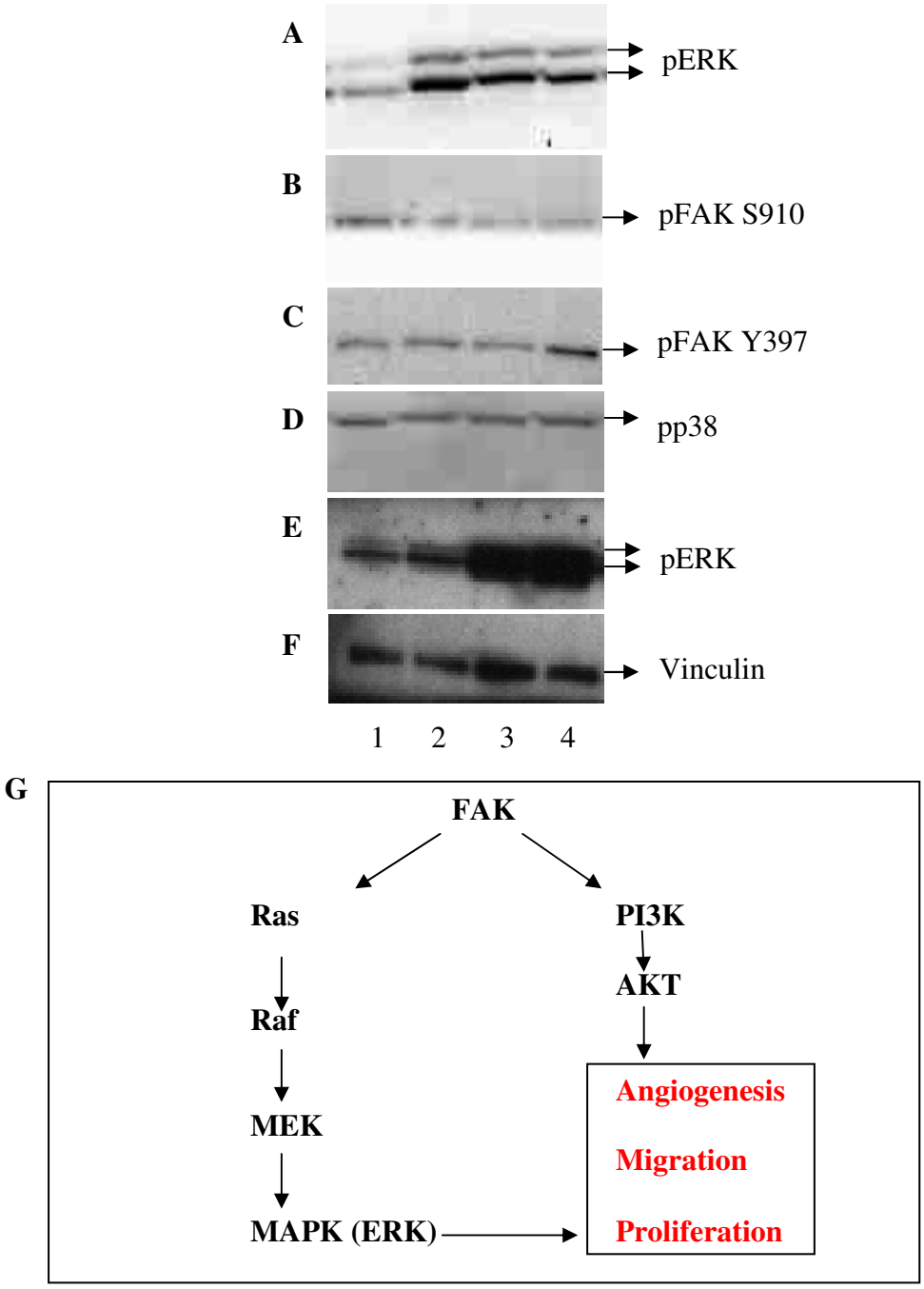
Figure 4.2. Endothelioma migration is not influenced by HPSE-degraded HS but FGF2 stimulates wound healing. A. To study how exogenous addition of HS will influence endothelioma (b.End3) biological activity, we added HPSE-digested bovine kidney HS to serum-free endothelioma medium. HS at the concentration range of 0-100 ng/ml did not have any effect on migration. B. Dose-dependent effect of FGF2 on endothelioma migration (Fig. 4.2B). At low concentration (1 and 10 ng/ml), FGF2 stimulated migration compared to no FGF2 treatment and at higher concentration (100 ng/ml), it did not affect cell migration. C. Shows photographs taken at T_0 and T_8 hours (melanoma) to explain how the assay works.


A
Relative Gap Closure (%)
16
12
8
4
0
0 ng/ml
1 ng/ml
10 ng/ml
100 ng/ml
HS (HPSE)


B
Relative Gap Closure (%)
16
12
8
4
0
0 ng/ml
1 ng/ml
10 ng/ml
100 ng/ml
FGF2



Relative Gap Closure = [1-(T_8/T_0)]

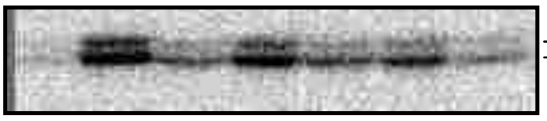


Fgure 4.3. Phosphorylation of ERK is stimulated by FGF2 and VEGF in endothelioma. A-D. FGF2 at the concentration range of 0 (lane 1), 1 (lane 2), 10 (lane 3) or 100 (lane 4) ng/ml stimulates phosphorylation of ERK but not of FAK or p38. E. VEGF at the same concentration range stimulates phosphorylation of ERK. F. Vinculin as loading control for E. G. Crosstalk between various signaling molecules. Depending on the cellular system, these molecules can also act independent of each other.

effects were seen at 10 and 100 ng/ml (Fig 4.3E). Since phospho-ERK is difficult to strip off the PVDF membrane; we used the top half of the same membrane to probe for the cytoskeletal-associated protein vinculin to serve as a loading control (Fig. 4.3F). Next, we used the melanoma cell surface HSGAG isolated by HPSE degradation in the signaling experiments to look for changes in phosphorylation of ERK in the presence or absence of 1 ng/ml FGF2 in endothelioma. At low HS concentrations (1-10 ng/ml), FGF2 phosphorylated ERK compared to no FGF2 treatment, but HS at a higher concentration (100 ng/ml) decreased this effect (Fig. 4.4).

We sought to investigate if removal of endothelioma cell surface HS by bacterial heparitinase III (Hep III, EC 4.2.2.8) treatment would influence FGF2-mediated endothelioma migration and signaling. We treated the b.End3 cell line with 10 µg/ml of Hep III for 1 h 37^{o}C at 50 rpm which is the optimum degradation time for Hep III. Enzymatic removal of cell-surface HS by Hep III treatment was incomplete after one hour incubation which was confirmed by flow cytometry (Fig 4.5A). We next treated the cells with heparitinase overnight but, digestion of cell-surface HS was still incomplete. Following treatment, cells were washed three times to remove any residual enzyme from the culture plates and the migration and signaling experiments were repeated. Even with partial removal of cell-surface HS, the endothelioma migration responded to exogenously added FGF2 (Fig 4.5B and C). FGF2 influenced the migratory behavior (Fig 4.5B) and phosphorylation of ERK (Fig 4.5C) as were already seen in the previous experiments.

We also treated the endothelioma cells with various concentrations of HPSE (0-1000 ng/ml) overnight to study if HPSE digestion would make any difference in FGF2 or VEGF-mediated signaling in these cells as were seen with melanoma in our previous study (Reiland,

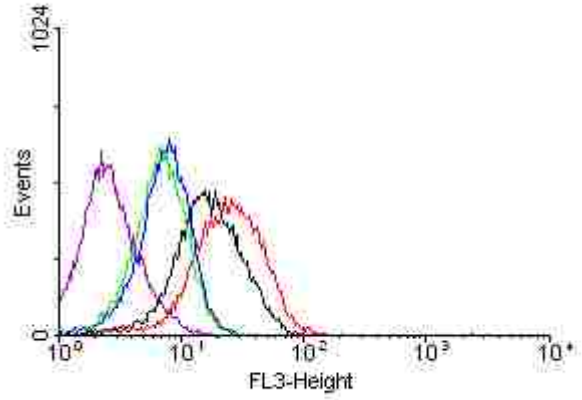


HS (B16B15b) ng/ml	0	1	1	10	10	100	100
FGF2 ng/ml	0	1	0	1	0	1	0

Fgure 4.4. Phosphorylation of ERK in endothelioma is not influenced by HPSE-degraded melanoma cell-surface HS but FGF2 stimulates signaling. A. To study how exogenous addition of HS will influence endothelioma (b.End3) signaling, we added indicated concentration of HPSE-digested melanoma cell-surface HS with or without FGF2 (1 ng/ml). HS at the concentration range of 0-10 ng/ml did not have an effect but FGF2 stimulates signaling as expected at these concentrations. However, 100 ng/ml of HS decreased this FGF2-mediated effect.

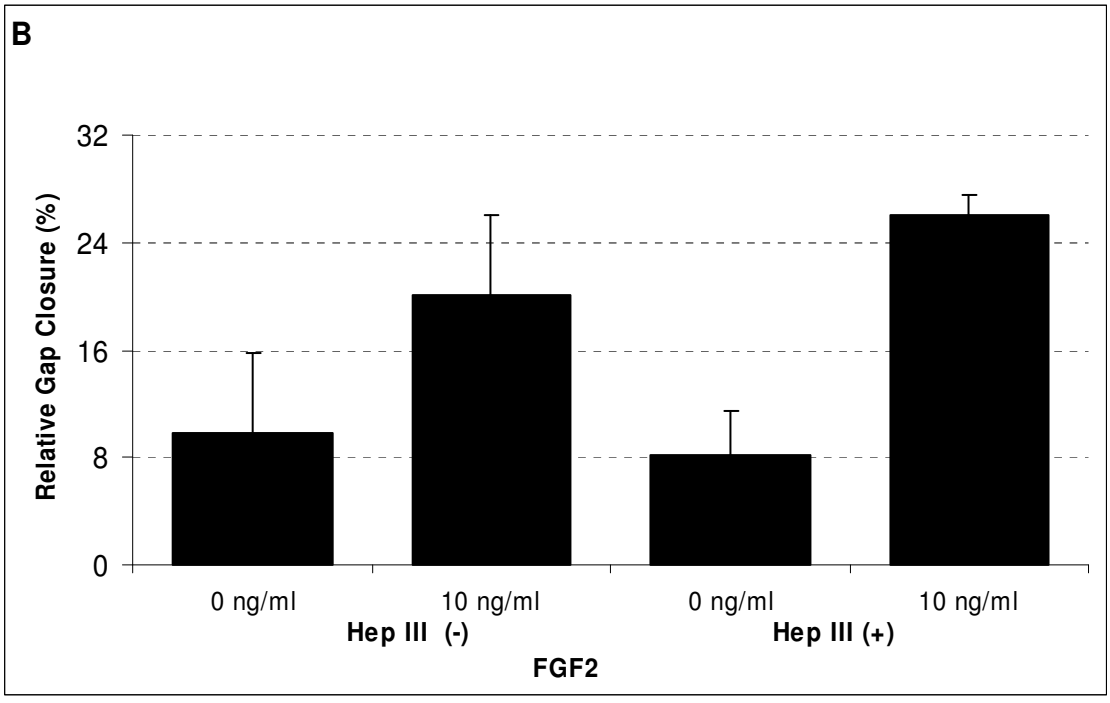
Figure 4.5. Enzymatic degradation of cell-surface HS either by Hep III or HPSE does not abolish endothelioma migration or signaling. A. b.End3 cells were treated with Hep III (10 µg/ml, black) or none (red) for 1 h at 37^{o}C at 50 rpm. Enzymatic removal of cell-surface HS was confirmed by flowcytometry with appropriate controls (blue and green, secondary and tertiary antibody control; purple, no antibody control). Following heparitinase treatment, cells were washed three times to remove any residual enzyme from the culture plates and migration (B) as well as signaling (C) in response to FGF2 was assayed. Even with partial removal of cell-surface HS, FGF2 influenced both the migratory behavior (B) and phosphorylation of ERK (C) in the endothelioma as were seen already in the previous experiments. D. The endothelioma cells were treated with various concentrations of HPSE (ng/ml) (lanes 1-3:0, lanes 4-6:10, lanes 7-9:100, lanes 10-12:1000) overnight to study if HPSE digestion would make any difference to mock (lanes 1,4,7,10) or FGF2 (lanes 2,5,8,11)-/VEGF (lanes 3,6,9,12)-mediated signaling in these cells, and again, unlike as seen with melanoma in our previous study (Reiland et al. 2006), HPSE treatment did not affect either FGF2 or VEGF-mediated signaling in the endothelioma.

A

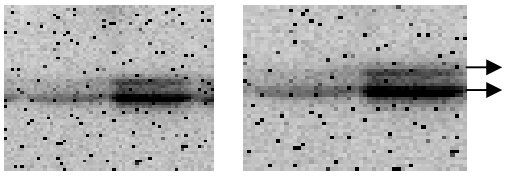


Relative Fluorescence Intensity (HS)

B

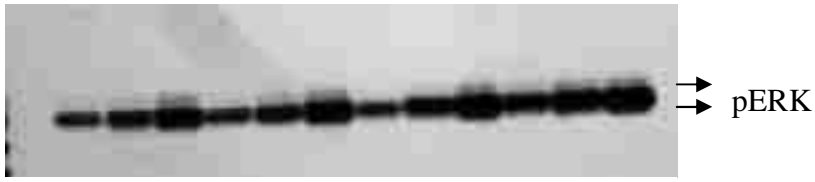


C



FGF2 (ng/ml) 0 10 0 10
Hep III (-) (+)

D



Kempf et al. 2006). HPSE treatment did not abolish either FGF2 or VEGF-mediated phosphorylation of ERK in the endothelioma (Fig 4.5D).

HPSE-Degraded Cell-Surface HSGAG Modify Melanoma Migration

To directly investigate whether the B16B15b cell-surface HSGAG were biologically active, we tested their effects on melanoma cell migration. The B16B15b cells possess aggressive migratory behavior. When HSGAG were added externally, there was a further increase in migration by 30% compared to no treatment (Fig. 4.6A). Addition of VEGF (0-100 ng/ml) did not augment this effect compared to control in at least two different experiments. Interestingly, VEGF did not influence migration even when added with HSGAG in comparison to HSGAG alone (Fig. 4.6A). This was unexpected since VEGF is known to require HS to exert its biological effects.

We also investigated effects on melanoma migration by adding fibroblast growth factor-2 (FGF2), platelet derived growth factor (PDGF) and interleukin 8 (IL8) and did not see any response. Interestingly, again we did not see an added effect on migration when FGF2 was added to cells with HSGAG in comparison to HSGAG alone (Fig. 4.6B). As with VEGF, this was unanticipated since these growth factors are known to require HS to exert biological effects.

HPSE-Degraded Cell-Surface HSGAG Do Not Influence Melanoma Proliferation

We next explored the effect of B16B15b cell-surface HSGAG on melanoma cell proliferation to test if similar conditions used in wound healing assays would also affect cell proliferation. Proliferation of melanoma cells was 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. We did not see an effect on proliferation either by HSGAG or by VEGF on melanoma cell proliferation (Fig. 4.7). Thus, exogenous addition of melanoma cell-

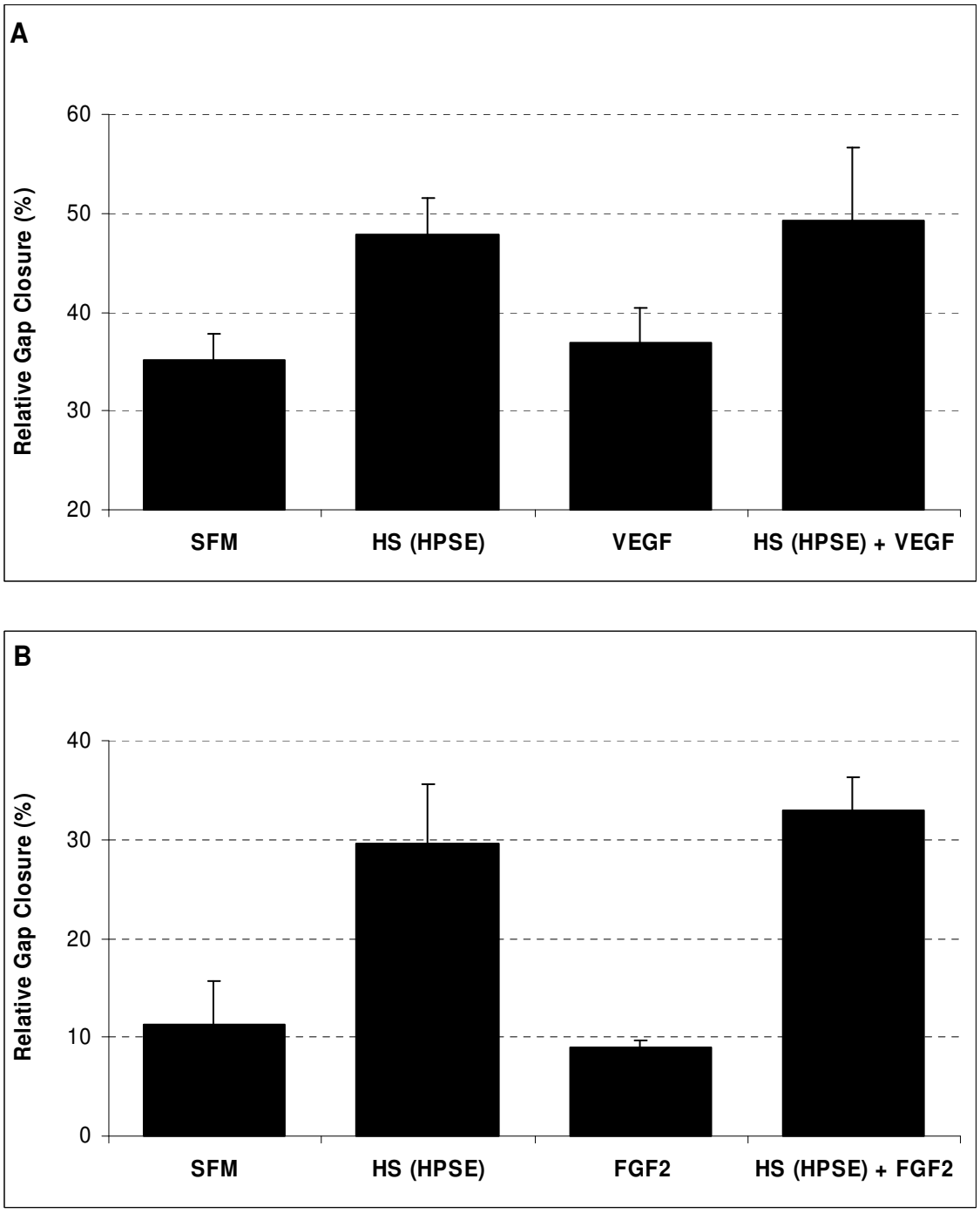


Figure 4.6. HPSE-degraded cell-surface HSGAG modify melanoma migration. When HSGAG were added externally to melanoma cells in wound healing assays, there was increased migration compared to control. Addition of VEGF (A) or FGF2 (B) did not affect migration with or without HS.

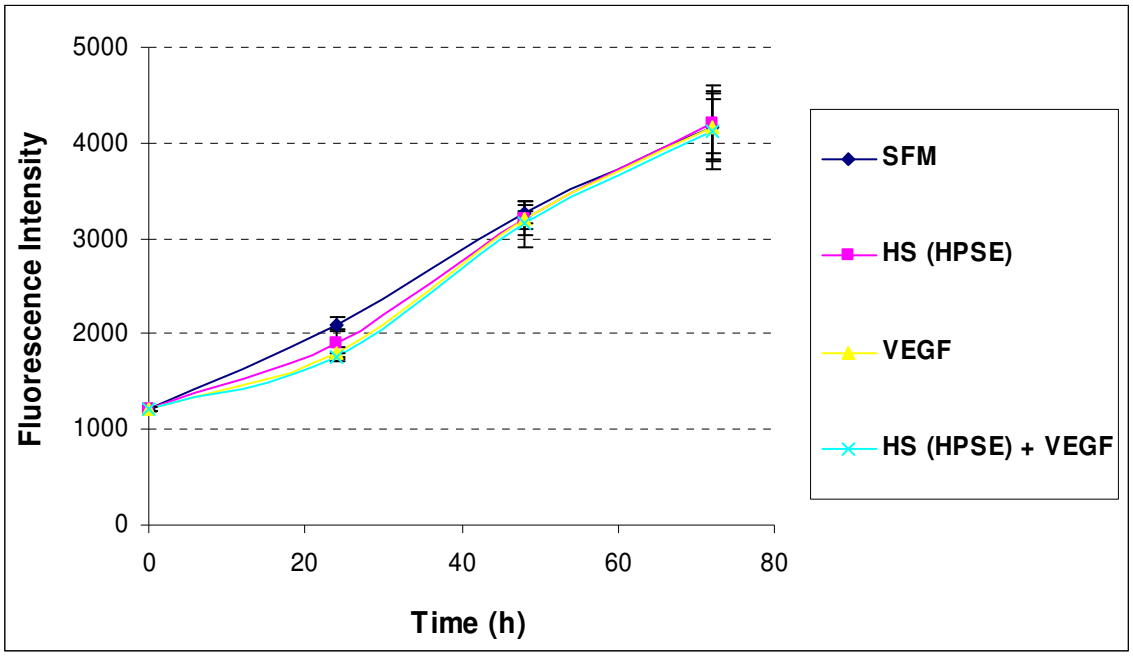


Figure 4.7. HPSE-degraded cell-surface HSGAG do not influence melanoma proliferation. Proliferation of melanoma cells were assayed by alamarBlue™, a non-toxic dye that monitors the reducing environment of the proliferating cell. Melanoma cell proliferation was monitored every 24 h for 72 h. Data are expressed as fluorescence emission intensity units as a function of time of incubation. Exogenous addition of VEGF or melanoma cell-surface HS isolated by HPSE treatment did not influence melanoma proliferation in at least three independent experiments.

surface HS isolated by HPSE treatment influences melanoma cell migration but does not affect cell proliferation.

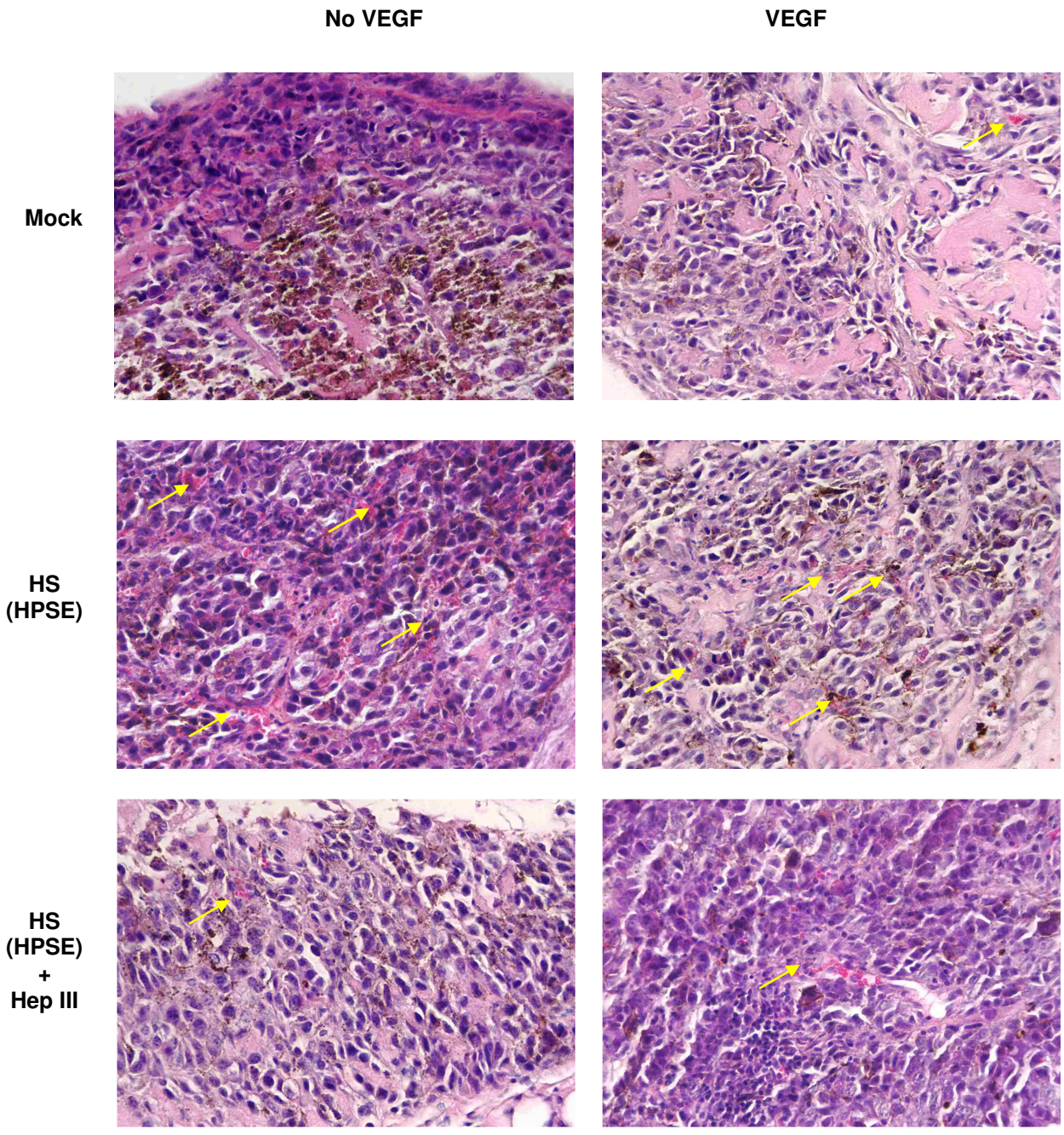
HPSE-Degraded Cell-Surface HSGAG Promote Angiogenesis *In Vivo*

Matrigel™ plug assay was performed to study the effect of B16B15b cell-surface HSGAG on angiogenesis *in vivo*. B16B15b cells were resuspended in reduced-growth factor Matrigel™ with or without HPSE-degraded melanoma cell-surface HSGAG and 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 HSGAG with (right) or without (left) VEGF. Group B received HPSE-treated HSGAG that were further treated with Hep III to cleave them into inactive disaccharide fragments with (right) or without (left) VEGF. Group C received melanoma cells in mock buffer with (right) or without (left) VEGF. Mice were sacrificed on the 10th day post-injection; tumors were excised, fixed in 10% formalin, and embedded in paraffin. Tumor sections (7 μm thick) were then H&E-stained to examine for blood vessel formation under the microscope. Blood vessel density was assessed by counting vessels within the tumor region in five different sections in each tumor.

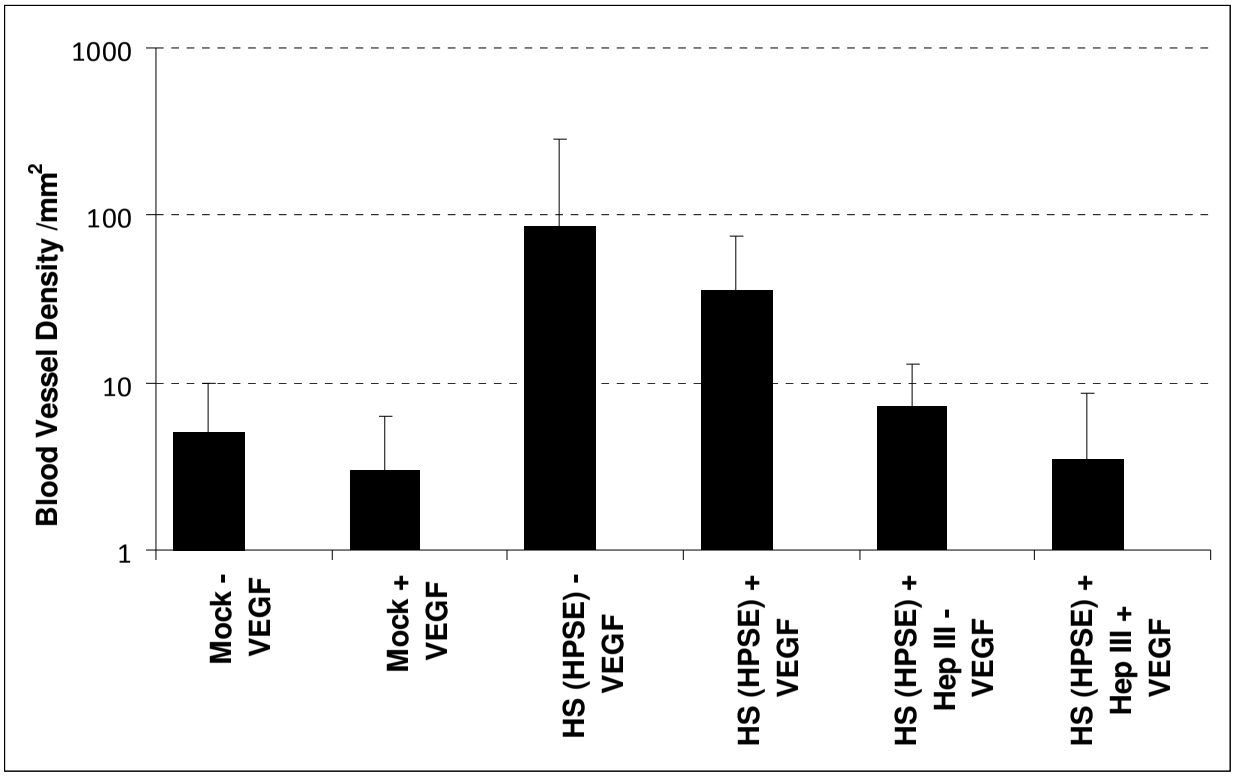
HPSE-treated cell-surface HSGAG induced a significant increase in intra-tumor blood vessel formation (Fig 4.8) in animals in group A compared to mock (group C) or Hep III treatment of HPSE-treated cell-surface HSGAG (group B) ($p < 0.0001$). Interestingly, the formation of increased numbers of tumor vessel was VEGF independent. Presence of VEGF did not affect angiogenesis in any of the three groups ($p = 0.2$-0.8). Notably, the absence of blood vessels inside the tumors led to areas of necrosis because of lack of nutrition and oxygen (Fig 4.8).

Figure 4.8. HPSE-degraded cell-surface HSGAG promote angiogenesis *in vivo*. Blood vessel density was assessed by counting vessels within the tumor region in five independent 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 HepIII 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.

A



B


1000
100
10
1
Blood Vessel Density /mm2
Mock - VEGF
Mock + VEGF
HS (HPSE) - VEGF
HS (HPSE) + VEGF
HS (HPSE) + Hep III - VEGF
HS (HPSE) + Hep III + VEGF

Structural Analyses of HPSE-Degraded Melanoma Cell-Surface HSGAG

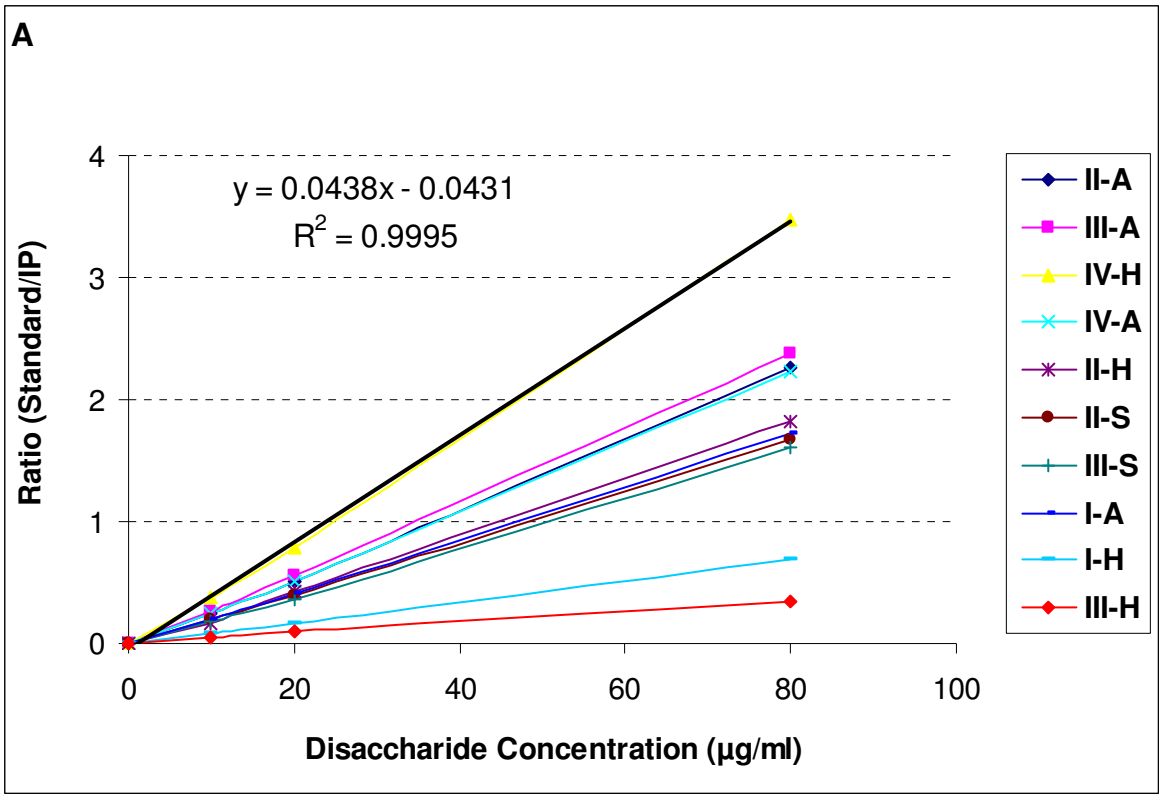
Isolated B16B15b melanoma cell-surface HSGAG were digested into disaccharides with 5 μg/ml of each of heparin lyases I, II and III at 37°C overnight. As a positive control, we used bovine kidney HS (50 μg) digested into disaccharides with 5 μg/ml of each of the heparin lyases overnight. Mock digestions were performed on bovine kidney HS in the heparin lyase digestion buffer. Finally, the 5μg/ml of each of heparin lyases in the heparinase digestion buffer was used as a negative control. Heparin disaccharide standards at 0, 10, 20, 40, and 80 μg/ml were run before and after the samples to control for variation and method reproducibility (Fig. 4.9A). Separation of the variously sulphated Δ-disaccharides was carried out by ion-pair reversed-phase chromatography (see methods and materials) (Karamanos, Vanky et al. 1997).

Disaccharides were recognized in the bovine kidney HS containing sample. We identified disaccharide peaks IVH, IVA, IIA, IIS, and, IIIS in this sample (Fig 4.9B). The heparin lyase non-treated HS did not have any recognizable disaccharide peaks, as expected (Fig. 4.9C). We did not identify any disaccharide peaks in the sample containing cell-surface HSGAG isolated by HPSE treatment except internal standard IP. The heparin disaccharide standards were identified at concentrations as low as 10 μg/ml. However, the signal was not very strong. Signals for IH and IIIH were low even at concentrations as high as 80 μg/ml (Fig. 4.9D). Remaining disaccharide standards generated stronger signals than those of IH and IIIH starting at 20 μg/ml (Fig. 4.9E). Hence, the HPLC method we used to characterize the fragments was not sensitive enough to generate any signal from the low concentration of cell-surface HSGAG.

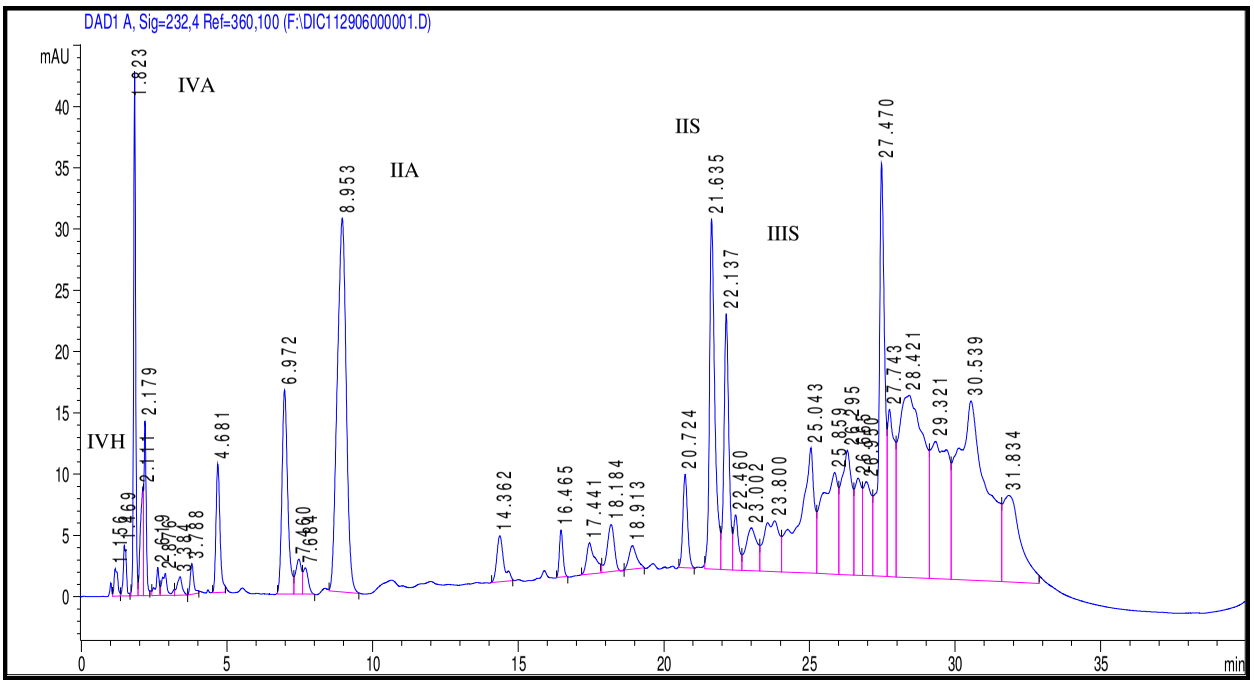
DISCUSSION

In the present study, we have investigated the role of HPSE-degraded cell-surface HSGAG in melanoma tumorigenesis. Our findings suggest that melanoma cell surface HSGAG

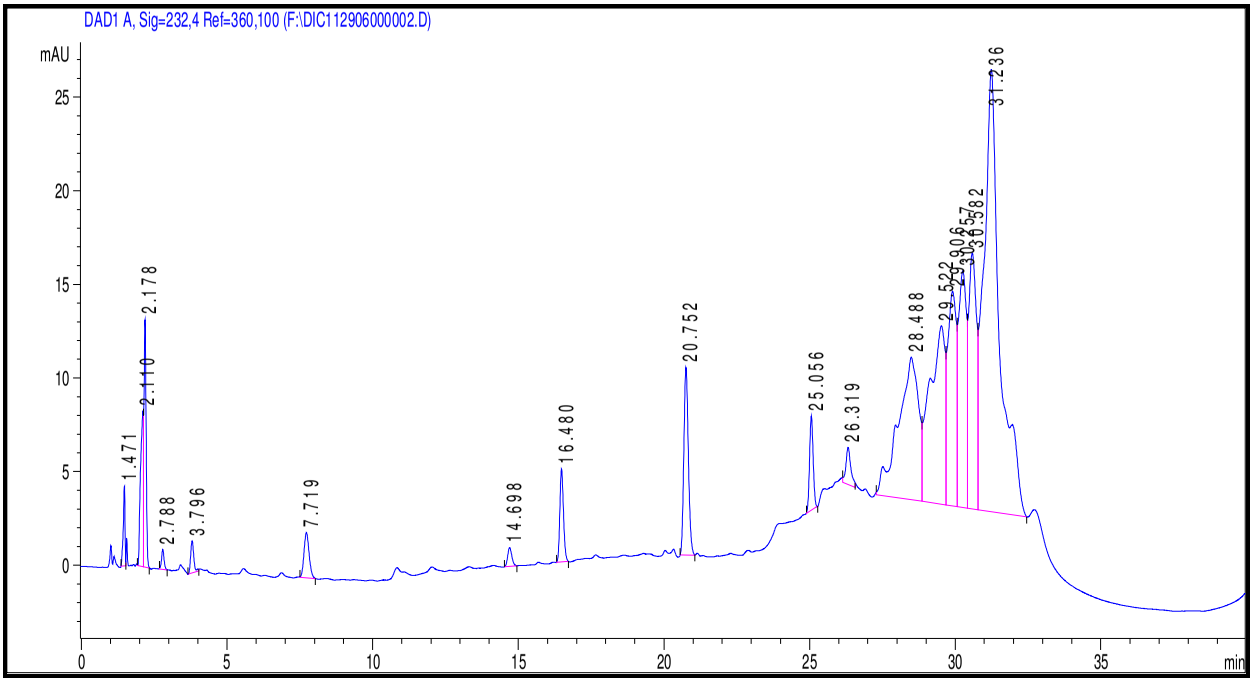
Figure 4.9. Structural analyses of HPSE-degraded melanoma cell-surface HSGAG. Separation of the variously sulphated Δ-disaccharides was carried out by ion-pair reversed-phase chromatography. Heparin disaccharide standards at 0, 10, 20, 40, and 80 µg/ml were run before and after the samples to control for variation and method reproducibility (A). Disaccharides were recognized in the bovine kidney HS containing sample; we identified disaccharide peaks matching standards IVH, IVA, IIA, IIS, and, IIIS in this sample (B). The heparin lyase-non treated HS did not have any recognizable disaccharide peaks (C). We did not identify any disaccharides peaks in the sample containing cell-surface HSGAG isolated by HPSE-treatment, data represents at least two different experiments (D). The heparin disaccharide standards at concentration of 80 µg/ml (E).


A
y = 0.0438x - 0.0431
R2 = 0.9995
Ratio (Standard/IP)
Disaccharide Concentration (µg/ml)
0
1
2
3
4
0
20
40
60
80
100
II-A
III-A
IV-H
IV-A
II-H
II-S
III-S
I-A
I-H
III-H

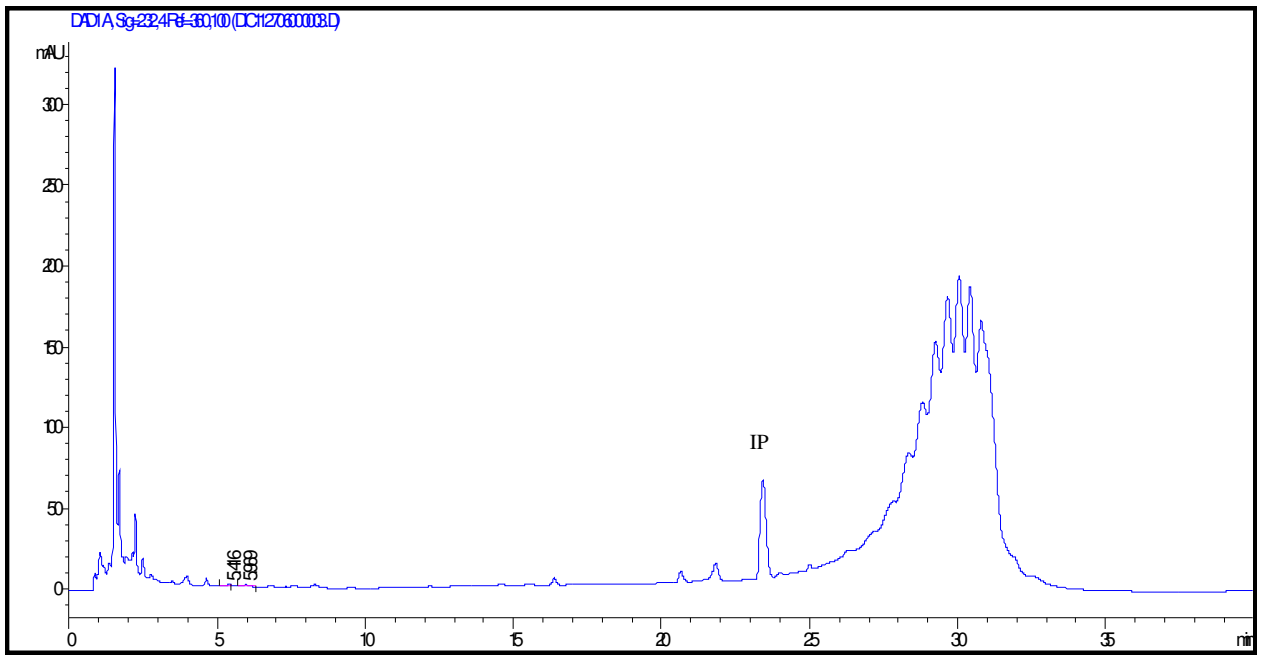
B


DAD1 A, Sig=232,4 Ref=360,100 (F:\DIC112906000001.D)
mAU
IVH
IVA
IIA
IIS
IIIS
1.823
2.179
2.111
4.681
6.972
8.953
14.362
16.465
17.441
18.184
18.913
20.724
21.635
22.137
22.460
23.002
23.800
25.043
27.470
27.743
28.421
29.321
30.539
31.834
min

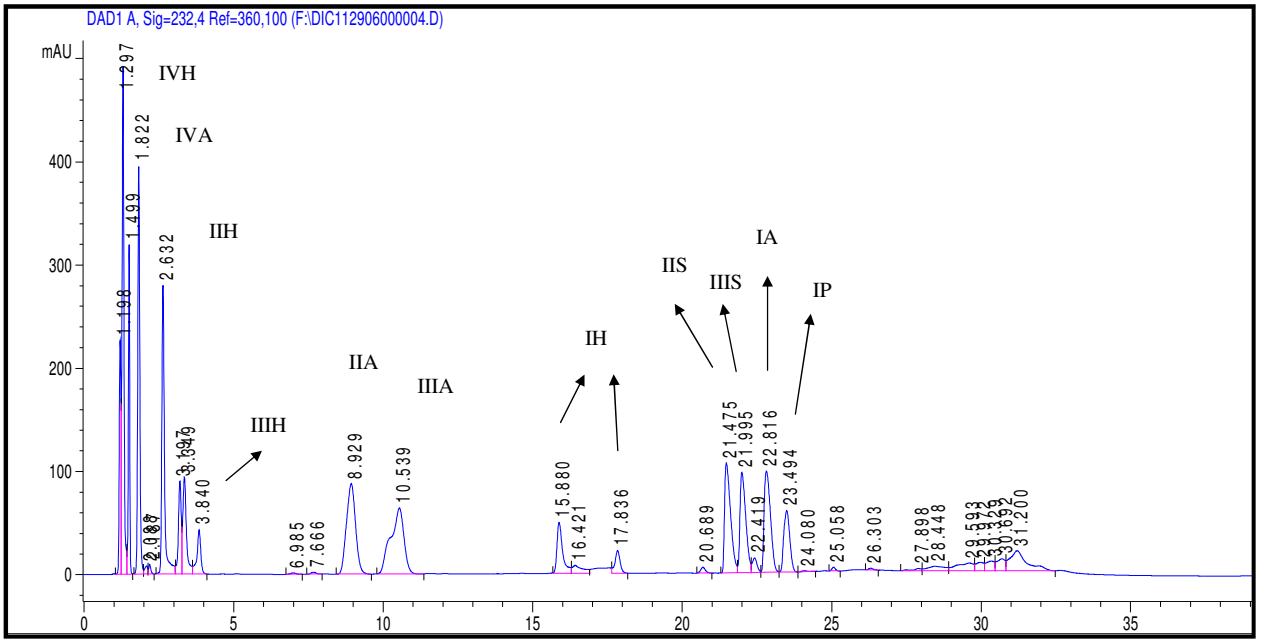
C


DAD1 A, Sig=232,4 Ref=360,100 (F:\DIC112906000002.D)
mAU
1.471
2.110
2.178
2.788
3.796
7.719
14.698
16.480
20.752
25.056
26.319
28.488
31.236
min

D


mAU
IP
min

E


DAD1 A, Sig=232,4 Ref=360,100 (F:\DIC112906000004.D)
mAU
IVH
IVA
IIH
IIIH
IIA
IIIA
IH
IIS
IIIS
IA
IP

isolated by HPSE treatment promotes melanoma migration and angiogenesis independent of VEGF activity. These results also provide direct 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 that can possibly stimulate melanoma tumorigenesis without the support of any growth factor or chemo/cytokines.

HSPG (Iozzo 2001) are currently recognized as cell surface/ECM active biological modulators (Kjellen and Lindahl 1991), and their degradation at the level of HS chains by glycosidases has significant regulatory consequences in cancer metastasis (Liu, Shriver et al. 2002a; Liu, Shriver et al. 2002b). Cell-surface/ECM HSGAG are complex polysaccharides that are ubiquitous in nature and are characterized by repeating disaccharide units of uronic acid (either iduronic acid or glucuronic acid) linked to a glucosamine (Gallagher 2001). The immense structural diversity of HSGAG allows them to bind and interact with numerous proteins including growth factors, chemokines, morphogens, and enzymes. The specificity of growth factor binding and interactions is dependent on the HSGAG sequence including their sulfation pattern, spacing of binding sites, and the three-dimensional structure of the HSGAG chain (Rapraeger 1995; Sasisekharan, Shriver et al. 2002; Robinson, Mulloy et al. 2006). HSGAG present on tumor cells also contain bioactive sequences that may affect tumor-cell phenotype in relation to cell growth and metastasis (Sasisekharan, Shriver et al. 2002; Liu, Shriver et al. 2002a; Roy 2007). 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 that mediate HS synthesis within the Golgi (Esko and Selleck 2002). On the other hand, it has also been shown that besides the modification that occurs in the Golgi apparatus

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) that remove 6-*O* sulfation on the HS (Dai, Yang et al. 2005; Narita, Staub et al. 2006) and, HPSE which cleaves HS into small, biologically active fragments (Nakajima, Irimura et al. 1983; Nakajima, Irimura et al. 1988; Dempsey, Brunn et al. 2000).

In non-mammalian systems, HS-degradative enzymes, such as bacterial heparitinases, heparinases from Flavobacterium heparinum, or endoglucosaminidases, cleave HS to disaccharides and tetrasaccharides, which are far too short for growth factor and ECM ligand binding (Ernst, Langer et al. 1995; Ernst, Venkataraman et al. 1996; Liu, Shriver et al. 2002a). Conversely, HPSE is a mammalian endo-β-D-glucuronidase that cleaves HS at specific intrachain sites, resulting in fragments of appreciable size (10–20 sugar units) (Nakajima, Irimura et al. 1984; Nakajima, Irimura et al. 1988; Marchetti 1997; Vlodavsky, Goldshmidt et al. 2001). Elevated levels of HPSE are known to be associated with brain-metastatic melanoma (Marchetti, Menter et al. 1993; Marchetti, McQuillan et al. 1996; Toyoshima and Nakajima 1999; Marchetti and Nicolson 2001; Marchetti 2002). 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 functions, including migration, adhesion, inflammation, angiogenesis, embryogenesis, and metastatic invasion (Parish, Coombe et al. 1987; Vlodavsky, Bar-Shavit et al. 1991; Hulett, Freeman et al. 1999; Kussie, Hulmes et al. 1999; Vlodavsky, Friedmann et al. 1999; Uno, Fujiwara et al. 2001; Vlodavsky and Friedmann 2001; Kelly, Miao et al. 2003; Edovitsky, Elkin et al. 2004; Reiland, Sanderson et al. 2004; Sasaki, Higashi et al. 2004; Roy, Reiland et al. 2005).

When overexpressed, HPSE increases tumor cell invasiveness *in vitro* and *in vivo* (Uno, Fujiwara et al. 2001; Roy, Reiland et al. 2005); conversely, downregulation of HPSE by anti-sense or siRNA decreases tumorigenesis both *in vitro* and *in vivo* (Uno, Fujiwara et al. 2001; Edovitsky, Elkin et al. 2004; Roy, Reiland 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. In turn, this may lead to subsequent inhibition of biological effects (Zetser, Bashenko et al. 2003; Reiland, Kempf et al. 2006).

Accumulating evidence suggests that cellular function and phenotype are highly influenced by the composition and size of HSGAG chains on HSPG (Perrimon and Bernfield 2000; Liu, Shriver et al. 2002a). A cell can respond to its microenvironment in markedly different ways by dynamically regulating the HS structure on its cell surface as insoluble HS, and in the ECM as soluble HS (Liu, Shriver et al. 2002a; Reiland, Kempf et al. 2006). HSPG and HS chains are present on the surface of all eukaryotic cells, including tumor cells and cells that are important for tumor survival such as the endothelial cell area surrounding a growing tumor, where HS can participate in the process of angiogenesis. This led us to propose that the pro-angiogenic activity of HPSE could partly be due to generation of bio-active 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 HSGAG since they express large amounts of it on the cell surface. The extent of HS degradation on B16B15b by HPSE was assessed by detection of cell-surface HS on FACS analysis. We were able to remove melanoma (B16B15b) cell-surface HS with HPSE treatment in a dose-dependent manner (Fig. 4.1A). Hydrolase action of HPSE introduces a water molecule to the uronic acid-

glucosamine sequence producing a glucuronic acid at the newly formed reducing terminus. In our cells, the mAb 3G10 epitope recognized the residual cell-surface HS following HPSE digestion even though an unsaturated uronic acid of HS is required for 3G10 reactivity (David, Bai et al. 1992). Possibly because 3G10 is an IgM and therefore pentameric, epitope recognition may be highly dependent on the organization of HS on the cell surface. Other groups have also detected total HS in nucleus using the 3G10 epitope with strong reactivity (Schubert et al., 2004). As expected, the HPSE-degraded cell-surface HSGAG migrated faster than non-treated bovine kidney HS (4.1B).

To directly test whether the B16B15b cell-surface HSGAG were biologically active, we tested their effects on melanoma and endothelioma biological activity. We also reasoned that since VEGF is a HS-binding growth factor and is essential for brain metastasis formation (Yano, Shinohara et al. 2000), these fragments would participate in VEGF-mediated activities. VEGF also influences a number of pathological conditions that involve abnormal angiogenesis, such as rheumatoid arthritis and diabetic retinopathy (Carmeliet 2000). A single VEGF gene is expressed as a number of VEGF isoforms through alternate mRNA splicing that differ by the presence or absence of heparin-binding domains (HBDs) encoded by exons 6 and 7 of the VEGF gene (Houck, Ferrara et al. 1991; Tischer, Mitchell et al. 1991). Among the most commonly known isoforms, VEGF121 is secreted in the ECM in a free form whereas VEGF189 is sequestered in the extracellular matrix (Plouet, Moro et al. 1997). VEGF165 is the most widespread and abundantly expressed splice variant that interacts with HSPG and neuropilins in a biologically active form and is found in both secreted and HS-bound form (Plouet, Moro et al. 1997).

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; Folkman, Merler et al. 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 (Hanahan and Folkman 1996; Iozzo and San Antonio 2001). Therefore, we decided to study the effects of HPSE-degraded HS in an endothelial system. We first investigated changes in migratory properties in murine brain endothelioma cell line b.End3, since migration is a critical event in angiogenesis (Carmeliet 2000). HPSE-degraded bovine kidney HS or cell-surface HS did not have any effects on b.End3 endothelioma cell migration or signaling (Fig. 4.2, 4.4), 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. The disaccharide sequence GlcA-GlcNS3S occurs predominantly in the endothelial cell system and mast cells, and this unit is vital in the pentasaccharide sequence for binding to antithrombin (Bourin and Lindahl 1993; Rosenberg, Shworak et al. 1997) while kidney HS contains a large amount of IdoA2S-GlcNS3S (Edge and Spiro 1990). Attempts to remove endothelioma cell-surface by HS enzymatic degradation of Hep III or HPSE did not alter the response to growth factor in this system which could be due to the fact that removal of cell-surface HS was incomplete. Hence, the remaining HSGAG on the cell-surface were enough to mediate growth-factor mediated signaling (4.5) which is known to occur (Krufka, Guimond et al. 1996).

VEGF165 binding to VEGFR1 is dependent on cellular HSPG because exogenous heparin/HS is unable to compensate for the loss of cell-surface HS, suggesting that HSPG play a

role in VEGF165 presentation to cell-surface receptors containing signaling domains (Terman, Khandke et al. 1994; Cohen, Gitay-Goren et al. 1995). VEGF165 is able to bind VEGFR2 in the absence of HS, but this interaction is improved by cellular or exogenous heparin/HS (Terman, Khandke et al. 1994; Gitay-Goren, Cohen et al. 1996). This suggests that HS on the HSPG regulates the interaction of VEGF to VEGFR2 and consequent biological activity (Schlessinger, Lax et al. 1995). In our system, however, the presence of VEGF165 did not influence the biological activities of the HPSE-degraded melanoma cell-surface HS including migration (Fig. 4.6) and proliferation (Fig. 4.7) of melanoma *in vitro*, and angiogenesis *in vivo* by Matrigel™ plug assay (Fig. 4.8). Carboxylate groups and 2-*O*-, 6-*O*-, and *N*-sulfation of HS promote VEGF165 interaction with its receptor; of these sites, 6-*O*-sulfates appears to be particularly important (Robinson, Mulloy et al. 2006).

A recent report by Robinson et al. 2006 demonstrated that VEGF requires highly sulfated sites on the HS for binding. These sites were exposed by enzymatic action of K5-lyase on HS as it demonstrated significant VEGF165 affinity. In contrast, cleavage of HS by heparinases or HPSE severely reduced VEGF165 binding (Robinson, Mulloy et al. 2006). This may explain the reason we failed to observe any biological effect with exogenously added VEGF and HPSE-degraded HS in melanoma. Another possibility could be that the basal level of activity that we observed was due to either VEGF binding to only endogenous cell-surface HS, or, autocrine VEGF produced by the melanoma cells saturated this effect. However, melanoma cell-surface HSGAG were able to stimulate melanoma migration (Fig. 4.5) and angiogenesis (Fig. 4.7) compared to the controls, suggesting that these fragments are tumorigenic even if they do not affect proliferation of melanoma cells (Fig.4.6). This finding is further strengthened by the fact that, following Hep III digestion, these fragments lose their pro-angiogenic effects.

Remodeling of the ECM and BM is vital for 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 2005). 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 pro-angiogenic growth factors, hence 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, including HPSE (Parish, Freeman et al. 2001; Sanderson 2005). Characterization of these HPSE-degraded melanoma cell-surface HS would potentially be useful. Our attempts to do so failed since the method we used did not have high sensitivity to low amounts of HSGAG present in the sample (Fig 4.8). The other methods that are available are also mostly optimized to use with large quantities of sample. Our future plans are to devise a method that would isolate individual oligosaccharides which can then be tested for pro-angiogenic/pro-tumorigenic properties. Identifying the oligosaccharide HS sequences will also lead to characterization of the yet unknown binding motifs for various growth factors such as FGF2 and VEGF that interact with HS. 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.

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CHAPTER V
CONCLUDING REMARKS

SUMMARY

In the first part of these studies it was demonstrated that HPSE is an important molecular determinant of metastasis. Down regulation of HPSE by an adenoviral antisense construct (Ad-AS/hep) decreased its expression and activity that correlated with invasive cellular properties *in vitro* and *in vivo*. Ad-AS/hep treatment of the human brain-metastatic melanoma cells (70W) inhibited HPSE-mediated invasion as indicated by the Matrigel^{TM} invasion assay *in vitro*, and decreased lung tumor colonization compared to control *in vivo* in nude mice (Roy, Reiland et al. 2005). When injected in the internal carotid artery, we observed a complete inhibition of brain tumor formation following Ad-AS/hep treatment in nude mice. Unexpectedly, overexpression of HPSE by Ad-S/hep also inhibited brain tumor formation possible due to extensive degradation of cell surface HS which in turn modifies growth factor activity (Reiland, Kempf et al. 2006). Briefly, the results by Reiland et al. (2006), suggested that at low concentration (which mimics the physiological concentration of HPSE *in vivo*), HPSE promotes melanoma tumorigenesis by stimulating FGF2-mediated signaling and activity, but at higher concentration it can also inhibit metastasis by attenuating FGF2-mediated signaling and activity in melanoma through extensive remodeling of the cell-surface HS (Reiland, Kempf et al. 2006).

Finally, cell-surface heparan sulfate is also known to inhibit or promote tumorigenesis depending on size and composition. We proposed that HPSE generates bioactive HS chains from the melanoma cell-surface that modify biological activities associated with vascular endothelial growth factor (VEGF), a molecule essential for brain metastasis. HPSE-degraded melanoma cell-surface HS stimulated migration but not proliferation of melanoma *in vitro*; it

also enhanced angiogenesis *in vivo*, independent of VEGF 165. This is in agreement with that current report by Robinson et al. (2006) which demonstrated that VEGF requires highly sulfated sites on the HS for binding and that cleavage of HS by heparinases or HPSE severely reduced VEGF165 binding (Robinson, Mulloy et al. 2006). This may explain the reason we failed to observe any biological effect with exogenously added VEGF and HPSE-degraded HS in melanoma. Another possibility could be that the basal level of activity that we observed was due to either VEGF binding to only endogenous cell-surface HS, or, autocrine VEGF produced by the melanoma cells saturated this effect. Since, the melanoma cell-surface HSGAG were able to stimulate melanoma migration and angiogenesis in comparison to the controls, it indicated that these fragments are tumorigenic even if they do not affect proliferation of melanoma cells. This finding is further strengthened by the fact that, following Hep III digestion, these fragments lose their pro-angiogenic effects. Interestingly, HPSE-treated melanoma cell-surface HSGAG did not have any effect on endothelioma *in vitro*. This could be due to tissue-specific HS structural differences present between the systems (Esko and Lindahl 2001).

We also attempted to characterize the melanoma cell-surface HSGAG isolated by HPSE degradation by ion-pair high pressure liquid chromatography. However, the method was not sensitive enough to detect nanogram quantities present in our samples.

Overall, these results suggested that HPSE is an important molecular determinant of melanoma metastasis, and that in addition to remodeling the ECM and releasing growth factors and chemokines from the cell-surface, HPSE contributes to the aggressive phenotype of melanoma by releasing bioactive HS from the melanoma cell-surface that can stimulate melanoma migration and angiogenesis.

CURRENT AND FUTURE RESEARCH CHALLENGES

While considerable progress has been made during the last three decades in understanding HPSE biology, there is much more to be learned. Regulation of the enzyme is very tightly controlled under physiological conditions (Parish, Freeman et al. 2001). There is convincing evidence that shows that the enzyme is upregulated in primary and metastatic human tumors and this correlates inversely with poor survival rate of cancer patients (Parish, Freeman et al. 2001; Vlodavsky and Friedmann 2001). However, how this enzyme is de-regulated in pre-cancerous lesions or in cancer is not fully understood. The exact mechanism by which it facilitates tumor progression is also not known. Despite the fact that its pro-angiogenic property is the most accepted mechanism behind HPSE-mediated tumor progression, this requires further confirmation. In the brain-metastatic melanoma as well as a glioma model, overexpression of HPSE protein led to inhibition of tumorigenesis suggesting a more complicated role of HPSE in the metastatic cascade. Interestingly, selective expression of nuclear HPSE in breast and esophageal carcinoma leads to cell differentiation indicating that nuclear HPSE expression could be used as a prognostic marker (Nobuhisa, Naomoto et al. 2007; Ohkawa, Naomoto et al. 2004). A few studies have revealed some of the mechanisms that induce HPSE protein expression during pathological conditions, but HPSE regulation at the transcriptional, translational and post-translational levels requires further investigation.

Additionally, development of a sensitive, high throughput activity assay or ELISA method to scrutinize HPSE levels and activity in body fluids of patients is needed in order to monitor response to therapy or to predict prognosis. It has been demonstrated that elevated levels of HPSE are detected in the plasma and/or urine of cancer patients as well as in other pathological disorders such as diabetes (Katz, Van-Dijk et al. 2002; Levidiotis, Freeman et al.

2004). HPSE is further involved in autoimmune disorders, diabetic nephropathy, and inflammatory disorders (Lider, Baharav et al. 1989; Levidiotis, Freeman et al. 2005; Edovitsky, Lerner et al. 2006) and we need to investigate the underlying mechanisms. HPSE is also implicated in neuro-degenerative diseases such as Alzheimer's disease (Bame, Danda et al. 1997). HPSE knock-out mice would be useful in this respect and are being generated by several groups to gain further insight into the mechanisms responsible in these normal and pathological processes. We also need a clear understanding of the 3D structure of the enzyme to develop and screen efficient inhibitory molecules. Currently, only a single active HPSE enzyme is known to be expressed by mammals, but recently a 55 kDa HPSE splice variant has been characterized that lacks enzymatic activity (Nasser, Avivi et al. 2007). The non-enzymatic functions of HPSE also point to new areas of interest, including identification of a putative HPSE receptor (Ilan, Elkin et al. 2006).

While HPSE itself is an attractive target for therapy, our lab and others have provided evidence that cell-surface HSGAG are bioactive and thus are another attractive target for therapy (Sasisekharan, Shriver et al. 2002; Liu, Shriver et al. 2002a). However, a major barrier to devising the HSGAG-related therapy had been the inability to sequence these molecules until recently (Karamanos, Vanky et al. 1997; Turnbull, Hopwood et al. 1999; Venkataraman, Shriver et al. 1999; Vives RR 1999; Liu, Shriver et al. 2002b; Saad, Ebel et al. 2005). The slow progress in sequencing technology was due to several reasons. There were very few tools including methods to purify the highly anionic HSGAG sequences, as well as few enzymes and chemical methods to cleave HSGAG precisely. Also, unlike DNA and proteins, there is no *in vitro* system available for amplifying HSGAG sequences of interest. All the available tools to sequence complex polysaccharides involve digestion of the HSGAG with bacterial heparitinases that

produce di- or tetra-saccharides. HPSE, however, produces oligosaccharides and therefore a method is needed to characterize the mixture of HPSE-digested oligosaccharides. Once the HPSE-generated fragments are isolated and sequenced, the individual fragments can be tested for biological activity. Fragments with the highest metastatic properties can be used to design inhibitory strategies which could be effective therapeutically. Furthermore, identifying the oligosaccharide HS sequences will also lead to characterization of the yet unknown binding motifs for various growth factors that interact with HS. Moreover, the design of unique agents targeted against these HS fragments can be an important addition to developing polysaccharide based anti-tumor therapy in melanoma.

FINAL COMMENTS

HPSE has essential pro-angiogenic and pro-metastatic roles, as well as important normal physiological functions such as neo-vascularization, inflammation, autoimmunity, and embryonic implantation attributed to its enzymatic activity (Ilan, Elkin et al. 2006). HPSE functions by degrading cell-surface and ECM HS and by releasing HS-bound angiogenic growth factors (i.e., FGF2, VEGF) from the ECM (Folkman, Klagsbrun et al. 1988) that facilitate proliferation, migration, invasion and metastasis (Ilan, Elkin et al. 2006). Furthermore, HPSE degraded bio-active HS fragments can stimulate angiogenesis by modifying activities of angiogenic growth factors (Reiland, Kempf et al. 2006). HPSE activity correlates with the metastatic potential of tumor cells; i.e., sub-lines with higher metastatic potential exhibit a higher enzymatic activity than low- or non-metastatic cells (Hulett, Freeman et al. 1999; Vlodavsky, Friedmann et al. 1999; Yang, MacLeod et al. 2005). Overexpression of HPSE by transfection or an adenoviral gene delivery method increases invasive and metastatic properties in cancers of esophagus, lung and in melanoma (Uno, Fujiwara et al. 2001; Roy, Reiland et al. 2005).

Conversely antisense, siRNA and ribozyme technologies-mediated reduction of HPSE expression leads to a decrease in invasive properties *in vitro* and *in vivo* (Uno, Fujiwara et al. 2001; Edovitsky, Elkin et al. 2004; Roy, Reiland et al. 2005). Interestingly, exceedingly elevated HPSE expression levels can also inhibit tumorigenesis, probably due to extensive cell-surface remodeling (Zetser, Bashenko et al. 2003; Reiland, Kempf et al. 2006). Additionally, selective expression of nuclear HPSE in breast and esophageal carcinoma leads to cell differentiation which could be used as a prognostic marker in cancer patients (Nobuhisa, Naomoto et al.2007; Ohkawa, Naomoto et al. 2004).

HPSE also exerts non-enzymatic activities, independent of its involvement in ECM degradation and remodeling. Inactive HPSE expressed on the cell-surface leads to firm cell adhesion, indicating an involvement in cell–ECM interaction (Goldshmidt, Zcharia et al. 2003). Inactive HPSE also enhances endothelial cell migration and invasion (Gingis-Velitski, Zetser et al. 2004).

While we are only starting to appreciate that cells dynamically regulate their HSGAG to alter intracellular signaling pathways in highly specific ways, new technologies that have already been developed to analyze and synthesize complex polysaccharides are unveiling the importance of glycobiology in cancer pathogenesis (Sasisekharan, Shriver et al. 2002). A better understanding of the structure–function relationships of these HSGAGs will help understand how they control various aspects of cancer. 'Glycoprofiling' or mapping out cell-surface HSGAGs on cancer cells possibly could lead to development of prognostic tools and diagnostic screens for cancer detection and staging. Identifying the HSGAG profile of a tumor might also predict its growth and metastatic potential, along with the tumors likely response to therapy (Sasisekharan, Shriver et al. 2002). Finally, the design of novel agents targeted against these HS

fragments could be a new cancer therapy. These molecules also might potentially escape immune surveillance in the patients and thus have a long-term therapeutic effect.

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VITA

Madhuchhanda Roy got a degree in Medicine from Nilratan Sircar Medical College in Calcutta (Kolkata), India, in 1997. After completion of one year of rotational internship in the departments of Medicine, Surgery, Gynecology & Obstetrics and Community Medicine in 1998, she moved to Bombay (Mumbai) for residency program in the departments of Medicine, Oncology and Intensive Care in Bombay Hospital till 2002. She joined the doctoral. program at the Louisiana State University (LSU), Baton Rouge, Louisiana, in Fall of 2002, in Dr. Dario Marchetti's tumor biology laboratories at the LSU School of Veterinary Medicine through the Department of Comparative Biomedical Sciences to begin her career as a scientist. She wants to apply her knowledge of medicine and basic sciences towards developing novel anti-cancer therapy. The degree of Doctor of Philosophy will be awarded at the Spring Commencement, 2007.