The role of heparanase in malignant melanoma and the development of a brain slice model to study mechanisms of brain metastatic melanoma in vivo

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THE ROLE OF HEPARANASE IN MALIGNANT MELANOMA AND THE DEVELOPMENT OF A BRAIN SLICE MODEL TO STUDY MECHANISMS OF BRAIN METASTATIC MELANOMA IN VIVO

A Thesis
Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science in The Interdepartmental Program in Veterinary Medical Sciences through the Department of Comparative Biomedical Sciences

by Brian P. Murry
B.S., Louisiana State University, 2002
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ABSTRACT

Heparanase (HPSE-1) is an endo-β-D-glucuronidase that cleaves heparan sulfate chains of proteoglycans (HSPG), and its expression has been associated with increased growth, mutations, and angiogenesis of tumors. Because metastatic melanoma cells express high levels of HSPG, and their tumors grow highly vascularized, we analyzed HPSE-1 expression in vivo by analyzing both experimental animals as well as clinical specimens with metastatic melanoma. Laser capture microdissection microscopy was used to isolate and extract melanoma cell populations from normal tissue. There was a 29-fold upregulation of heparanase expression, detected by real-time PCR, by metastatic melanoma cell populations compared to normal lung tissue in experimental animals. Additionally, immunohistochemistry (IHC) revealed a selective HPSE-1 staining in human metastatic melanoma when compared to primary melanoma tumors. IHC also showed a marked staining for HPSE-1 around blood vessels and vascular regions. Finally, we developed an orthotopic brain slice model that should aid in the study of brain cancer metastasis. This model allows for the characterization of tumor invasion at both quantitative and qualitative levels. Brain-metastatic melanoma cells when treated with HPSE-1 versus untreated cells showed a significant increase in the number of cells that invaded. Secondly, melanoma cells, showed increasing HPSE-1 protein expression during a time course experiment. We concluded that HPSE-1 expression likely plays important roles in regulating the growth and progression of melanoma. These results also further emphasize the potential importance of therapies designed to block HPSE-1 activity in controlling this type of cancer.
CHAPTER 1: INTRODUCTION

Cancer is the second leading cause of death, and is a major health concern in the United States and in the world. Cancer metastasis, which is the spread of tumor cells from a primary site of origin to distant organs within the body, is a particularly important area in neoplasia as it accounts for 90% of cancer related mortalities (Bogenrieder et al., 2003). The brain is one of several highly targeted areas for metastatic cancers, and one of the highest frequencies of brain metastases is found in malignant melanoma (Graf et al., 1988). Malignant melanoma has the unfortunate distinction of being the cancer type with the highest increase in frequency of occurrence, especially among young adults. Mechanisms responsible for melanoma progression to highly aggressive metastatic disease are not fully understood (Bogenrieder et al., 2003).

Although metastatic mechanisms are not completely known, it has been demonstrated that metastasis is not a random event. Rather, it is the result of a complex sequence of events that occurs between normal host cells, tissues, and malignant cells (Bogenrieder et al., 2003; Nakajima et al., 1988). Notably, all cells that metastasize must intravasate from the primary tumor site into the blood vessels, circulate through the blood, attach to vessel walls, exit the vasculature, and grow in the new surrounding tissue (Nicolson, 1982). During the metastatic process, invading tumor cells must have the ability to breach tissue barriers. Degrading barriers such as basement membranes, extracellular matrices (ECM), and connective tissue is dependent on proteolytic enzymes such as heparanase (Nakajima et al., 1988).

One of the main components of the ECM are heparan sulfate proteoglycans (HSPGs), and macromolecules composed of a core protein with heparan sulfate chains (HS) covalently attached to it. These proteoglycans are ubiquitous in the ECM and as cell surface molecules anchored into the plasma membrane. Recently, it has become increasingly clear that heparan
sulfate (HS) and heparan sulfate proteoglycans (HSPGs) play important roles in promoting tumor growth and metastasis. The main focus of this project was based on tumor invasiveness by the action of heparanase (HPSE-1), an enzyme, which plays a central role in tumor progression by releasing angiogenic molecules within the ECM resulting from the degradation of HSPGs.

HPSE-1 is a recently cloned enzyme, the first and only example of purification and cloning of a mammalian HS degrading enzyme (Hulett et al., 1999; Kussie et al., 1999; Toyoshima et al., 1999; Vlodavsky et al., 1999). HPSE-1 is involved in the degradation of the cell surface and ECM (Bernfield et al., 1999; Iozzo, 1998; Iozzo, 2001; Rapraeger, 1995; Sanderson, 2001). HPSE-1 is an endo-β-D-glucuronidase which cleaves heparan sulfate proteoglycans (HSPG).

HPSE-1 has significant regulatory consequences in cancer metastasis, and its expression has been associated with increased growth, metastasis, and angiogenesis of tumors (Vlodavsky et al., 2001).

The objective of this project was to establish 1) the expression and functionality of HPSE-1 in malignant melanoma in vivo, and 2) to develop an organotypic culture system that may be useful in studying the process of brain-metastatic melanoma invasion. This model provides a better representation than the currently available model systems of the ECM molecules normally encountered by invading melanoma cells. This brain slice model gives the ability to monitor tumor invasion at both quantitative and qualitative levels. For example, similar orthotopic brain slice models are currently being used as invasion assays for glioma cells and monitoring dendrite development (Nakada et al., 2004; Salama-Cohen et al., 2005)
CHAPTER 2: LITERATURE REVIEW

2.1 Historical Overview

Metastasis, which is the spread of cancer cells that have originated from a primary tumor of origin to distant loci throughout the body, is a major area of interest within the cancer research field. The reason for this high degree of interest resides in the fact that metastasis is the leading cause of cancer related mortalities. Cancer metastasis accounts for 90% of the deaths of a disease that is the second leading cause of death in the United States. This makes understanding the metastatic process at the systemic, cellular, and molecular levels important goals of cancer research (Bogenrieder et al., 2003; Marchetti et al., 2003a).

Over 130 years ago, Stephen Paget published the ‘seed and soil’ hypothesis as an explanation for non-random patterns of metastatic development. This hypothesis has stood the test of time and is still one of two main models for the patterns of metastasis. Paget’s hypothesis was centralized around the idea that metastasis was not due to chance. Instead it was the result of certain tumor cells (Paget called them the “seed”) that had an affinity for certain organs (Paget related as the “soil”). His model was that metastasis formation is dependent upon both “the seed and the soil” being compatible (Fidler et al., 2002).

The other model for the spread of malignancy, proposed by James Ewing in 1932, states that metastasis occurs solely by a mechanical process. He stated that metastasis was the result of anatomical and hemodynamic factors of the vasculature. Although circulatory anatomy may indeed influence the dissemination of many malignant cells, it can not fully explain the patterns of metastatic development since there is also evidence that tumor cell properties may determine the outcome of malignancy (Hart et al., 1980).
It is important to point out that Paget’s or Ewings hypotheses should not be defined as mutually exclusive; rather they are both correct and overlapping to a certain extent.

Cancer metastasis is a process that consists of sequential and independent steps, and studies have shown that neoplasms go through a series of changes from the original benign tumor into a lethal malignant disease state. For instance, tumors can originally be benign and then transform into a malignancy. This has been attributed to the fact that these cells have generated genetic variants in order to proceed through the metastatic cascade. These genetic variations lead to tumor transformation and proliferation. Together with proliferation comes the need for development of blood vessels and extensive vascularization (named angiogenesis) in order to maintain tumor viability (Folkman, 1986). Next, the malignant cells detach from the primary tumor and intravasate into blood vascular and lymph systems where they adhere to and arrest in the vasculature of distant target organs. These malignancies then extravasate through the blood vessel walls where they establish a tumor-host microenvironment which ultimately leads to cell proliferation and metastasis formation (Fidler et al., 2002).

Although the mechanisms for metastasis are not well understood, it is known that proteolytic enzymes play a role in the degradation of the ECM, which is essential for intra- and extravasation of tumor cells and the formation of metastasis to distant organs (Liotta, 1986). Therefore, a review of the role that proteolytic enzymes (specifically heparanase) and co-factors play in cancer metastasis development is important.

2.2 Brain-Metastatic Melanoma

Malignant melanoma also represents one of the best characterized solid tumors with respect to tumor invasion and metastatic spread (Hofmann et al., 2005). Usually the fatal step in the progression of many malignant cancers is often tumors metastasizing to the brain. The
frequency of melanoma occurrence and the morbidity of brain metastasis alone would be sufficient reason for alarm, but when one combines these two factors, they make the area of brain-metastatic melanoma a particularly important biological and clinical problem (Nicolson et al., 1996).

The brain provides a unique target for malignant melanoma by being confined to the skull. These brain metastatic tumor cells must penetrate a formidable blood brain barrier in order to invade the brain tissue. Malignant melanomas metastasize to the brain with one of the highest frequencies of any cancers that colonize the central nervous system (CNS) (Marchetti et al., 2003a). Metastases to other parts of the body are generally tolerated and asymptomatic, but due to the confined nature of the brain, tumor formation usually results in rapid decline in health (Menter et al., 1994). This morbidity is usually due to cerebral edema which is a major complication of CNS tumors. This build up of fluid on the brain is caused by the brain’s lack of a lymphatic drainage system, and this creates problems for a closed environment where homeostasis is important for normal brain function. These characteristics of the brain microenvironment make treatment both difficult and necessary (Sawaya et al., 1996; Soffietti et al., 2002).

2.3 The Role of the Extracellular Matrix in Cancer

It was originally thought that the extracellular matrix (ECM) surrounding tumor cells was an inert scaffold only providing for cell growth and differentiation. It is now apparent that the ECM plays essential roles in both normal and pathogenic cellular function and phenotype (Liu et al., 2002a). The current direction of thought is that the ECM is an active participant in cell signaling processes. This concept is based on the fact that ECM synthesis is highly regulated by the cell, which uses the ECM to modulate and process signals at the cell surface thereby
influencing cell function (Sasisekharan et al., 2000). The ECM is made of three major components: the first component is the structural proteins which help maintain morphology by providing tensile strength; the second component is proteoglycans which are protein core from which heparan sulfate glycosaminoglycan extensions protrude; and the last component is soluble signaling molecules like growth factor, cytokines, and chemokines that are bound to the ECM by the heparan sulfate glycosaminoglycan chains (HSGAGs) (Lin et al., 2000).

An important area of trying to determine the mechanism of cancer metastasis and development is the proteoglycan component of the ECM. There is much interest in proteoglycans because they are present both in the ECM and at the cell surface, thus they are directly involved in the cell-tissue interface. Proteoglycans are involved in both how the cell processes signals that arise from the ECM, and also, how a signaling molecule that is released from the cell diffuses to and is processed at a distant site (Liu et al., 2002a).

2.4 The Function and Structure of Proteoglycans

Proteoglycans are a superfamily of molecules that are distinguished by the covalent attachment of one or more glycosaminoglycan chains (GAG) to a polypeptide core. Differences in GAG composition are used to discern the four classes of GAGs that attach to core proteins: chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS)/heparin, and keratin sulfate (KS). The proteoglycans of interest are usually heparan sulfate proteoglycans (HSPGs) this is because cell suface proteoglycans primarily have HS attached to them. Virtually all cells express HSPGs at their cell surface, and a large number of genetic mutants that disrupt HS synthesis and modification demonstrate that HS has a critical role in controlling how cells interact with extracellular ligands (Kramer et al., 2003).
Two families of polypeptides appear to carry the majority of the cell surface heparan sulfate on mammalian cells: glypicans, which are attached to the plasma membrane via glycosylphosphatidylinositol (GPI) anchors, and syndecans, which are transmembrane proteins. Four syndecans and five glypicans, all encoded by separate genes, have been described to date (Bernfield et al., 1992). HSPG expression is developmentally regulated and altered in various pathological processes, including cancer. The expression patterns are believed to mirror those of ligands that require HSPGs to elicit their cellular responses. For instance, syndecan-1 cell surface expression is usually down-regulated in metastatic phenotypes. At the same time, syndecans are shed from the cell surface as soluble molecules that regulate protease and growth factor activity, and this soluble syndecan expression in tumors has been correlated with increased cancer progression and invasion (Sanderson, 2001).

2.5 The Structure and Variability of Heparan Sulfate Glycosaminoglycans (HSGAGs)

HSGAGs are ubiquitously expressed on the surface of every eukaryotic cell and have shown importance in the regulation of normal and pathological physiology (Sasisekharan et al., 2002). HSGAGs are synthesized in the Golgi apparatus beginning with four monosaccharides linking to the proteoglycan core protein, usually either a syndecan or a glypican. This chain is then turned into a polysaccharide by the sequential addition a glucosamine linked to either iduronic or glucuronic, and there are many possible disaccharide unit combinations for HSGAGs (Gallagher, 2001). These different possibilities 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 combinations because each site can be either sulfate or unsubstituted. There is also the opportunity 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. Lastly, the N-position of the glucosamine has three possible states in that it can be either sulfated, acetylated, or unsubstituted, which results in 48 different possibilities for disaccharide building blocks. This gives HSGAGs over 30 times as many possible variations as that of polypeptides and 10,000 times that of DNA, having the potential for a staggering degree of variation (Esko et al., 2001).

The result of HSGAGs having such a vast structural diversity is that they have the ability to bind and interact with a various array of proteins which includes growth factors, chemokines, morphogens, and enzymes. Growth factors, such as insulin like growth factor, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF), have shown to have important roles in the process of tumor development (Iozzo, 2001; Marchetti et al., 2003b; Nurcombe et al., 2000; Sasaki et al., 2004). The specificity of these interactions is dependent on HSGAG sequence, spacing of binding sites, and the three-dimensional structure of the HSGAG chain. These various HSGAG functions are important for both signaling molecules and enzymes to function in an appropriate manner.

2.6 HSGAGs in Cancer Development and Metastasis

HSGAGs have been shown to be involved in the transformation of a normal target cell to a cancer cell. It has been demonstrated that HSGAGs present on tumor cells contain bioactive sequences that effect several aspects of tumor-cell phenotype in relation to cell growth and metastasis. Tumor cell surface HSGAGs can promote growth factor signaling and tumor cell proliferation (Sasisekharan et al., 2002). The fibroblast growth factor receptor (FGF) family, which has diverse roles in regulating cell proliferation, migration, and differentiation when inappropriately expressed, can contribute to the pathogenesis of cancer. There are numerous levels of evidence implicating the differential ability of HSGAGs in regulating FGF signaling via
their cell surface tyrosine kinase receptors (FGFR). For example, it has been observed that the specificity of a given FGF to a FGFR is mediated by what appears to be distinct tissue-specific HSGAG sequences, and tumor cells have been reported to control the affinity that HSPGs and growth factors like basic fibroblast growth factor (bFGF) have for each other by altering the density and sulfation patterns of their HSPGs (Sasisekharan et al., 1994). The expression of and the structural integrity of HSGAGs on the cell surface have shown to change with the metastatic transformation of certain cell types (Kure et al., 1987). For example, it has been shown that B16 melanoma cells with high metastatic propensities have a lower concentration of cell surface HSGAGs than sublines with less metastatic potential. It has also been shown that highly metastatic B16 sublines degrade HSGAGs faster than sublines of lower metastatic spread (Nakajima et al., 1983).

HSGAGs are not randomly involved in cancer metastasis, rather they play intricate roles in this multi-step process. In order for a tumor to develop beyond a diameter size of 1-2 mm, an increase in vascularity is necessary, and HSGAGs are directly involved with the angiogenic process (Iozzo, 2001). In fact, bFGF has a dual role in its interaction with HSGAGs in that it is involved with tumor growth, and it is also involved with the development of new blood vessels. HSGAGs which are present on endothelial cells can indirectly effect tumor growth; they can either enhance or inhibit neovascularization by regulating signal transduction of bFGF or vascular endothelial growth factor (VEGF). In addition, HSGAGs have dual roles in tumor metastasis as well. Since HSGAGs are at the interface between tumor cells and normal host cells, it allows for them to mediate tumor and host cell interactions (Raman et al., 2003). HSGAGs regulate tumor metastasis to sites such as the brain by arbitrating interactions between cancer cells and platelets and endothelial cells and host organ cells (Varki et al., 2002). HSGAGs are
also involved in attempts to deter metastasis by acting like barriers in the ECM. This HSGAG barrier, however, is overcome by invading tumor cells that secrete degradative enzymes. Studies have shown a direct correlation between the production of heparanase, an enzyme that degrades the polysaccharide component of the ECM, and the invasiveness of tumor cells (Vlodavsky et al., 2001).

2.7 The Function of Heparanase in Tumor Progression and Metastasis

Heparanase (HPSE-1) is an enzyme that was first identified in the B16 murine metastatic melanoma cell sublines (Hook et al., 1975). It was shown in these B16 sublines that HPSE-1 correlated with lung metastasis potential (Nakajima et al., 1983). Since that time, HPSE-1 has been linked to a wide number of cancers including bladder, colon, gastric, breast, pancreatic, and brain (Friedmann et al., 2000; Gohji et al., 2001; Koliopanos et al., 2001; Marchetti et al., 2001; Maxhimer et al., 2002; Tang et al., 2002). This suggests that HPSE-1 plays a role in the sustained development and pathology of malignant diseases and would make a good potential target for cancer therapy (McKenzie et al., 2003).

Heparanase is an endo-β-D-glucuronidase that affects various biological processes through degradation of the ECM and release of HS-bound growth factors, cytokines, and enzymes. Heparanase participates in ECM desolation by invading cells, and HPSE-1 enzyme activity has been detected in several types of normal hematopoietic cells, including neutrophils, megakaryocytes, and activated lymphocytes and may mediate their extravasation during inflammatory and immune responses (Matzner et al., 1992; Nakajima et al., 1988; Schubert et al., 2004). Additionally, heparanase has been identified as a participant of embryogenesis (Revel et al., 2005), but is mainly known for its role in cancer invasion and metastasis. HPSE-1 does more than simply degrade and remodel extracellular matrix. HPSE-1, rather than fully digesting
HSGAGs, cleaves HSGAGs at only a few sites, producing fragments that are 10–20 sugar residues long. There is also evidence that the fragments of heparan sulfate generated by heparanase are more biologically active than the native HSGAGs from which they are derived (Sanderson et al., 2004). The human HPSE-1 gene is located on chromosome 4 and encodes a 543-amino-acid protein that has recently been purified and cloned (Hulett et al., 1999; Kussie et al., 1999; Toyoshima et al., 1999; Vlodavsky et al., 1999). This protein was originally synthesized as a 65 kDa glycosylated pro-enzyme that is activated by proteolytic cleavage to remove a linker region and form an active heterodimer of 8 and 50 kDa polypeptides (Fairbanks et al., 1999). The hydrolase activity of HPSE-1 works by cleaving the linkage between the glucuronic acid and N-acetylglucosamine of the HSGAGs (Nakajima et al., 1983). The HPSE-1 enzyme activity is closely regulated by pH, achieving maximal activity under acidic conditions at an optimum range between a pH of 5.5-8.8. This characteristic of HPSE-1 activity correlates well with the notion that the microenvironment in tumors is generally more acidic than in normal tissues. This acidity of the tumor microenvironment is found within hypoxic areas of growing tumors (Tannock et al., 1989). Upon activation in this acidic environment, HPSE-1 facilitates the release of several protein modulators that effect cell functions such as migration, adhesion, inflammation, and angiogenesis. HPSE-1 activation then facilitates processes which are all steps in the development of metastatic invasion (Hulett et al., 1999; Kussie et al., 1999; Nakajima et al., 1988; Parish et al., 1987; Toyoshima et al., 1999; Vlodavsky et al., 1999; Vlodavsky et al., 2001; Zetser et al., 2003).

2.8 Neurotrophins in Brain Metastasis and Heparanase Production

Metastasis is not a random event, and there are reasons that metastasis frequently occurs in certain organs. The reasons for these metastatic patterns may lie within the stromal cells and
the paracrine growth and differentiation factors that they secrete (Marchetti et al., 2004). The first one of these factors to be purified and characterized was nerve growth factor (NGF). NGF is important for the survival of and differentiation of neurons in both the sympathetic and central nervous system (Marchetti et al., 1993). NGF is the prototype of a family of neurotrophins (NT) which are a group of neurotrophic factors that are distinct from cytokines and FGFs. Although NGF is the best characterized NT, it is also known that other family members like brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) are also involved as neurotrophic substances as well. The biological effects of NTs are mediated through two unrelated classes of cell surface membrane receptors. All NTs can bind to p75 neurotrophin receptor (p75NTR) which is a low-affinity receptor. There is, however, a family of high-affinity Trk receptors that are bound selectively by certain NTs. For instance, NGF binds TrkA, BDNF and NT4/5 bind TrkB, and NT-3 binds TrkC (Chao et al., 2002). When NT receptors (NTR) are overexpressed in melanoma cells, they have shown to be more aggressive and have a stronger survivability than cells that express low NTR levels (Marchetti et al., 2004). Binding of NGF along with p75NTR being overexpressed has been characterized as giving cells both a survival advantage and creating aggressive malignant melanoma cells. There are previous studies that have shown that cells incubated with select NTs showed an increased ability to invade into a membrane like matrix, but what was of greater interest is the fact that these NTs caused an increase in HPSE-1 activity. The result is an NT-driven degradation of the ECM by HPSE-1 (Walch et al., 1999).
CHAPTER 3: SELECTIVE HEPARANASE LOCALIZATION IN MALIGNANT MELANOMA CANCERS

3.1 Introduction

Malignant melanoma has the unfortunate distinction of being the cancer type with the highest increase in frequency of occurrence, especially among young adults. Mechanisms responsible for melanoma’s progression to a 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 melanoma cells to invade distant organs (Bogenrieder et al., 2003). Heparanase (HPSE-1) plays a critical role in tumor progression by degrading the heparan sulfate (HS) chains of HS proteoglycans (HSPG) and by releasing angiogenic molecules stored within the ECM (Vlodavsky et al., 2001). HSPG are present on the cell surface and in the ECM of tissues and their degradation has significant regulatory consequences in cancer metastasis (Nardella et al., 2004). HPSE-1 is an enzyme that was cloned in 1999 (Hulett et al., 1999; Kussie et al., 1999; Toyoshima et al., 1999; Vlodavsky et al., 1999) as the first and thus far the only example of purification and cloning of a mammalian HS degradative enzyme. HSPE-1 acts as an endo-β-D-glucuronidase cleaving HS glycosidic bonds at specific intrachain sites resulting in the formation of fragments of appreciable size (10-20 sugar units) (Nakajima et al., 1984; Nakajima et al., 1988; Vlodavsky et al., 2001). Its hydrolase activity facilitates the release of several protein modulators that affect cell functions such as migration, adhesion, inflammation, embryogenesis, angiogenesis, and metastatic invasion (Hulett et al., 1999; Kussie et al., 1999; Marchetti et al., 1993; Marchetti et al., 1996; Nakajima et al., 1988; Toyoshima et al., 1999; Vlodavsky et al., 1999; Vlodavsky et al., 2001). Furthermore, elevated HPSE-1 mRNA levels correlate with reduced survival rates in post-

*Chapter 3 reprinted by permission of Professor D.A. Spandidos, the editor of International Journal of Oncology.
operative cancer patients (Vlodavsky et al., 1999; Vlodavsky et al., 2001). These observation led us to hypothesize that HPSE-1 would be substantially elevated in melanoma tissues particularly when compared to normal tissues from the same specimen. In the present report, we provide first evidence that 1) HPSE-1 mRNA is significantly upregulated in lung metastatic melanoma versus adjacent lung tissue; 2) HPSE-1 protein expression is significantly augmented in metastatic melanoma clinical specimens while mostly negative in primary tumors; and 3) HPSE-1 accumulates around blood vessels and vascular regions in brain-metastatic melanoma specimens.

3.2 Materials and Methods

3.2.1 Tissue Culture and Experimental Metastasis Assays

Early-passage murine invasive melanoma cells (B16F1, B16F10 and B16B15b) were maintained as monolayer cultures in a 1:1 (v/v) mixture of Dulbecco’s modified Eagle’s/ Ham’s F-12 medium (DMEM/F-12) supplemented with 5% fetal bovine serum (FBS) (v/v). Cells were maintained at 37°C in a humidified 5% CO2/95% air (v/v) atmosphere and were passaged using 2 mM Ethylene Diamine Tetra Acetic Acid (EDTA) in Phosphate buffered saline (PBS) before reaching confluence, as previously reported (Marchetti et al., 1993; Marchetti et al., 1996). Eight-week old female C57BL/6 mice were obtained from Harlan Sprague Dawley and quarantined for 1 week. The mice were randomly divided into three groups with four mice in each group receiving an injection of B16F1, B16F10, or B16B15b cells respectively. Injection in the lateral tail vein (I.V.) was carried out using 2 x 10^6 cells suspended in serum-free medium (200 µl). Mice were sacrificed after 17 days when they were moribund due to dyspnea.
3.2.2 Surgical Specimens

Specimens were obtained from the Cooperative Human Tissue Network, Tissue Procurement Facility, University of Alabama at Birmingham, AL, and from the Surgery Department at the University Hospital of Udine, Italy. These specimens were obtained frozen or were cut into 2x2x1 cm fragments, fixed in the 10% buffered formalin and paraffin embedded. Sections (5 µm thick) were cut into sialinized slides. Following treatment, slides were immunostained and examined in the Department of Pathobiological Sciences at the Louisiana State University School of Veterinary Medicine to identify tumor samples that contained metastatic melanoma as well as adjacent normal lung tissue. This resulted in the selection of 10 cases of brain-metastatic melanoma with adjacent brain tissue, 25 cases with metastatic melanoma to sites other than brain, and 10 cases of primary melanomas.

3.2.3 Tissue Preservation

Freshly dissected lungs were collected from mice, washed in cold diethylpyrocarbonate (DEPC)-treated PBS, and then incubated at 4°C on a rocking platform for 24 hrs in 30% sucrose (Parlato et al., 2002). The tissue was again rinsed with PBS and flash frozen in isopentone over dry ice. Frozen sections were embedded in optimal cutting temperature medium (OCT) and mounted on a cryomold. Sections were subsequently cut (10 µm thick) at -25°C, fixed in decreasing concentrations of ethanol (100 % EtOH, 70 % EtOH, and 50 % EtOH) for 20 sec each, stained for 20 sec with cresyl violet acetate, and then again dipped in 50 % EtOH, 70 % EtOH and in 100 % EtOH (two times). Slides were air-dried and stored at -80°C until used.
3.2.4 Laser Capture Microdissection Microscopy and RNA Extraction

Laser capture microdissection microscopy (LCMM) (Emmert-Buck et al., 1996; Fend et al., 1999; Sugiyama et al., 2002) was performed using the P.A.L.M. Laser-MicroBeam System (P.A.L.M., Wolfratshausen, Germany) following protocols provided by the manufacturer. The P.A.L.M. system was used to dissect normal tissue from tumorigenic tissue for the differential analysis of heparanase levels. Previously prepared slides were removed from -80°C just prior to use to avoid potential RNA degeneration. The P.A.L.M. system catapulted tissue (10^4 to 1.5 x 10^4 cells) into the lid of a reaction tube covered with extraction buffer (0.5 ml; Arcturus Inc., Mountain View, CA). The cap was inserted onto the reaction tube and the assembly was kept on ice. Following an incubation step at 42°C for 30 min, total RNA was extracted following the suggested protocol using the PicoPure RNA Isolation Kit (Arcturus Inc.). RNA was eluted using the kit elution buffer (18 µl). Presence of RNA was confirmed by PCR with the housekeeping gene GAPDH for murine samples and β-actin for human samples (Kreuzer et al., 1999; Overbergh et al., 1999).

3.2.5 cDNA Synthesis and PCR Amplification

cDNA synthesis was performed with a reverse transcription (RT)-mixture (25 µl; Promega, Madison, WI). First, RNA (13 µl) was incubated with 0.3 µg of oligo dTs (Invitrogen, Grand Island, NY) at 70°C for 5 min. Then, 5 µl of 5x RT buffer, 0.5 mM dNTPs, 25 units of RNAsin, and 200 units of M-MLV (Promega) were added, and the final RT-mixture was incubated at 42°C for 50 min with a heating step to 94°C for 2 min. PCR reactions were performed with a PCR-mixture (25 µl) consisting of cDNA (2 µl), 2.5 µl of 10x PCR buffer, 0.2 mM dNTPs, 0.5 µM primers, and 1 unit of Taq Polymerase (Promega). PCR was first performed with the housekeeping genes GAPDH (for murine samples) and β-actin (for human...
samples) to check for viable RNA. Samples testing positive for GAPDH or β-actin were tested for the HPSE-1 presence. Amplification was performed using the GeneAmp PCR-system 9700 (Applied Biosystems, Foster City, CA). Human samples had an initial denaturation step at 94°C for 5 min which was followed by 40 cycles of denaturation at 94°C for 30 sec and an annealing/extension step at 67°C for 60 sec for human β-actin or 60°C for 60 sec for human heparanase and murine GAPDH. The conditions for murine heparanase were 94°C for 5 min followed by 35 cycles at 94°C for 30 sec, 60°C for 45 sec, and 72°C for 45 sec with a final extension at 72°C for 10 min. Primer sequences and expected amplicon sizes are listed in Table 1. Agarose (1%) gel electrophoresis was subsequently performed.

3.2.6 Real-Time PCR

The fluorogenic probe used for real-time PCR analyses of murine heparanase contained the reporter dye FAM covalently attached at the 5’-end and a quencher dye TAMRA at the 3’-end (Applied Biosystems). The endogenous control used for murine samples in this study was 18S ribosomal RNA (Applied Biosystems), the reporter dye for 18S was VIC and the quencher dye was TAMRA. All fluorogenic probes were HPLC-purified. Analysis was performed using the ABI PRISM 7700 sequence detection system (Applied Biosystems). Amplification was carried out in a reaction volume (30 µl) that consisted of cDNA solution (2.5 µl) from the RT reaction, 2x TaqMan Mastermix (Applied Biosystems), 900 nM heparanase primer or 50 nM of 18S primer, and 200 nM of each probe in ddH2O. Amplification was performed in triplicate in MicroAmp optical tubes using the standard amplification protocol as recommended by the manufacturer.
3.2.7 Quantification of HPSE-1 mRNA Expression

Using the internal fluorogenic oligonucleotide probes, each well was monitored for fluorescent dyes, and signals were regarded as significant if the fluorescent intensity statistically exceeded (10-fold) the standard deviation of baseline fluorescence as Threshold cycle (C_T). C_T was defined as the cycle at which a significant increase in emission intensity was first detected. Relative mRNA quantitation was performed using 18S as the housekeeping gene and the comparative ΔΔC_T-method as previously described (Brink et al., 2000).

3.2.8 Immunohistochemistry for Murine Samples

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 were mounted on silanized slides (Fisher Scientific, Springfield, NJ) and deparaffinized. Endogenous peroxidase was blocked by incubating the sections in H_2O_2 (3.0%). After the blocking of nonspecific reactivity with rabbit serum for 30 min at room temperature (25°C), sections were incubated at 4°C for 60 min using HPSE-1 rabbit polyclonal antibody raised against human HPSE-1 and crossreacting with the murine enzyme (Pharmacia-Upjohn Inc., Kalamazoo, Michigan). Following rinsing, slides were incubated with biotinylated anti-rabbit IgG and then with Vectastain ABC reagent. Peroxidase activity was determined using the NovaRED substrate kit (Vector Laboratories Inc.), and the slides were counterstained with Mayer’s hematoxylin. As a negative control, sections were subjected to normal serum blocking with omission of the primary antibody.

3.2.9 Immunohistochemistry for Human Samples

Sections were prepared from human melanoma tissue samples which had been formalin-fixed and paraffin-embedded. The tissues were sectioned at 5 μm, mounted on silanized slides,
heated, deparaffinized, and rehydrated. Endogenous peroxidase was blocked with a H₂O₂ solution (3.0%). Slides were pretreated with proteinase K and then blocked with horse serum. Sections were incubated at room temperature (25°C) for 30 min using a mouse monoclonal antibody raised against human HPSE-1 (2 mg/ml, diluted 1:200 and 1:500) (Tsukuba Research Institute, Novartis Pharma K.K., Takarazuka, Japan). Additionally, a mouse monoclonal antibody directed against human endothelial marker CD34 was also used (Cell Marque, Hot Springs, AR). Following rinsing, slides were incubated with biotinylated goat anti-mouse IgG and then with Vectastain ABC reagent (Vector Laboratories). Peroxidase activity was determined using the NovaRED substrate kit (Vector Laboratories), and slides were counterstained with Mayer’s hematoxylin. Control sections were treated identically but an isotyped matched mouse monoclonal antibody was used as the primary antibody.

3.2.10 Statistical Analysis

Staining of each section was assigned scores ranging from 0 to 4+ as follows: negative (0), weakly positive (1+), moderately positive (2+), positive (3+), and highly positive staining (4+). For IHC determinations, positive cases were those that stained with an intensity score of ≥ 1, baseline mark for staining positivity.

All results comparing tumorigenic tissue versus normal tissue were analyzed using the paired Student’s t-test. They were considered significant if p < 0.05.

3.3 Results

3.3.1 Melanoma Development in Mice

To examine for HPSE-1 presence, mice were inoculated with B16F1, B16F10, or B16B15b melanoma cells and sacrificed 17 days later when they exhibited severe signs of dyspnea. Necropsy was performed with primary emphasis on lung tissue, and necropsy showed severe
infiltration of this tissue with black fragile nodules. Histological analyses confirmed the pulmonary nodules to be melanoma metastasis. A representative experiment showing of lung-metastatic melanoma in a mouse injected with B16F10 cells is shown (Figure 3.1).

3.3.2 Localization of HPSE-1 in Metastatic Melanoma Lesions

In order to obtain the precise RNA content of melanoma tissue, we employed laser capture microdissection microscopy (LCMM) to avoid contamination between cancerous and normal tissue samples. LCMM is beneficial because it allows for specific isolation of very small tissue samples (Emmert-Buck et al., 1996; Fend et al., 1999; Parlato et al., 2002; Sugiyama et al., 2002). Furthermore, LCMM makes real-time PCR (RT-PCR) and PCR analysis of very small tissue samples more feasible and reproducible. Melanotic and normal pulmonary cells could be efficiently isolated into different samples. Since the cancer metastasized to the lung, we used similar populations of normal lung tissues as controls for HPSE-1 analysis. The process of laser capture microdissection of melanoma tissue is shown in Figure 3.2.

In order to confirm that RNA extractions worked, PCR was performed on all samples as shown (Table 3.1). In murine samples, PCR was performed using GAPDH as the housekeeping gene. The GAPDH gene showed good RNA extractions in ten out of twelve samples, and samples that tested negative were from the two mice that died before they could be euthanized. Once the samples tested positive for the housekeeping gene, analysis for murine heparanase was performed. The PCR for murine heparanase demonstrated the expected 205 bp band in the tumor samples; however, all samples from adjacent normal tissue tested negative for HPSE-1 cDNA presence (Figure 3.3).

3.3.3 HPSE-1 mRNA is Upregulated in Murine Melanoma Tissues

Because HPSE-1 has been shown to be involved in cancer invasion, angiogenesis,
Figure 3.1 A Representative Experiment of Lung-Metastatic Melanoma. Murine malignant melanoma cells were injected intravenously (tail vain injection) in 6-week old female mice (C57BL/6). At the end of experimental period, mice were euthanized, and the lungs with metastatic melanoma were extracted and preserved for subsequent analysis.
Figure 3.2 Detection and Extraction of Melanoma Cells in Lung Tissues by LCMM. Sections before (A) and after (B) LCMM, showing the remaining cells on the slide. NLT, normal lung tissue. Melanoma cells are indicated.
Table 3.1 Sequences of PCR Primers and Sequence Specific Probes for Different Targets.

<table>
<thead>
<tr>
<th>Target Sequence</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Amplicon Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH(^a)</td>
<td>forward probe(^c) reverse</td>
<td>TTCACCACCATGGAGAAGGC TGCACTCTGCACCACCCAACTGCTTAG GGCATGGACTGTGGTCATGA</td>
<td>236</td>
</tr>
<tr>
<td>18S(^a)</td>
<td>probe(^d)</td>
<td>Supplied by Perkin Elmer (4308310)</td>
<td>187</td>
</tr>
<tr>
<td>HPSE-1(^a)</td>
<td>forward probe(^c) reverse</td>
<td>GACAAGGAACCGACTTCCGAAGAA Performed by Perkin Elmer CTGTAGAGCATGTCCACTGAGCTT</td>
<td>205</td>
</tr>
<tr>
<td>GAPDH(^b)</td>
<td>probe(^e)</td>
<td>Supplied by Perkin Elmer (402869)</td>
<td>226</td>
</tr>
<tr>
<td>β-actin(^b)</td>
<td>forward reverse</td>
<td>AGCCTCGCCTTTTGCCGA CTGGTGCTGGGGCG</td>
<td>180</td>
</tr>
<tr>
<td>HPSE-1(^b)</td>
<td>forward probe(^c) reverse</td>
<td>TCGTGGACCTGGACTTCTTCA CCACGGACCCCACGTTCTT ACAAGCCTCTTGCCAAGGTA</td>
<td>150</td>
</tr>
</tbody>
</table>

\(^a\)Sequence for murine sample  
\(^b\)Sequence for human sample  
\(^c\)FAM-labeled DNA probe  
\(^d\)VIC-labeled DNA probe  
\(^e\)JOE-labeled DNA probe
Figure 3.3  Agarose Gel Electrophoresis Showing PCR for Expression of Murine *heparanase* in Metastatic Melanoma and Adjacent Lung Tissues Obtained by LCMM.

(A) Lanes 1 to 8: melanoma tissue samples; Lane 9: negative control (H₂O); Lane 10: positive control (B16F10).  (B) Lanes 1 to 9: normal lung tissue samples; Lane 10: negative control (H₂O); Lane 11: positive control (B16F10).

and metastasis, we determined comparative and quantitative HPSE-1 mRNA levels in tumor
versus normal tissues. We performed real-time PCR to determine whether heparanase is upregulated in metastatic tissue versus normal tissues. The first choice as a murine housekeeping gene for sample normalization was murine GAPDH. With GAPDH as the housekeeping gene there was a marked difference in $C_T$ values between normal tissue and tumor tissue throughout all samples. This difference was up to 16 $C_T$ values difference between samples, which corresponds to a $2^{16}$ or 65,536-fold difference. For this reason, GAPDH could not be used for the housekeeping gene. Analysis of ribosomal 18S expression as a different housekeeping gene showed a similar result, but the trend was not nearly as profound. Data of quantitative expression analysis for HPSE-1 was normalized to the housekeeping gene 18S (Table 3.2). The melanoma samples after normalization had an average $\Delta C_T$ value of 10, and the average $\Delta C_T$ value for normal lung was 14.86 (Table 3.2). The average calculated $\Delta \Delta C_T$ value (normal minus melanoma) was 4.86, which corresponds to a 29-fold increase of heparanase mRNA expression in melanoma tissue (Figure 3.4). Results for $\Delta \Delta C_T$ were shown to be significant to $p < 0.05$. $C_T$ values for individual sample $\Delta C_T$'s are indicated in Table 3.2.

3.3.4 Elevated HPSE-1 Expression in Metastatic Melanoma Lesions

Immunohistochemistry was performed for the presence of heparanase with anti-HPSE-1-PAb on paraffin-embedded, lung-metastatic samples from mice. We observed an intense immunostaining for heparanase detected on the cell-surface and ECM for each of melanoma tumors samples. A representative experiment HPSE-1 immunostaining is shown in Figure 3.5. Immunostaining for HPSE-1 also tested positive in all human samples of metastatic melanoma. Conversely, primary melanoma tumors were mostly negative for HPSE-1. In metastatic melanoma to brain, 8 cases had very intense staining for HPSE-1 (Figure 3.6). Of note, HPSE-1
Table 3.2  Comparison of HPSE-1 mRNA in Adjacent Normal and Metastatic Melanoma Tissues Melanoma by Quantitative Real-Time PCR.

<table>
<thead>
<tr>
<th></th>
<th>Δ CT melanoma tissue</th>
<th>Δ CT normal tissue</th>
<th>ΔΔ CT</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse 1</td>
<td>6.8(^a)</td>
<td>14.0</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>Mouse 2</td>
<td>N.D.(^b)</td>
<td>15.0</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Mouse 3</td>
<td>9.1</td>
<td>13.9</td>
<td>4.8</td>
<td>Average</td>
</tr>
<tr>
<td>Mouse 4</td>
<td>9.9</td>
<td>15.7</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td>Mouse 5</td>
<td>15.5</td>
<td>16.0</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Mouse 6</td>
<td>N.D.</td>
<td>17.3</td>
<td>N.D.</td>
<td>Average fold</td>
</tr>
<tr>
<td>Mouse 7</td>
<td>9.5</td>
<td>10.1</td>
<td>0.6</td>
<td>difference</td>
</tr>
<tr>
<td>Mouse 8</td>
<td>10.0</td>
<td>14.0</td>
<td>4.0</td>
<td>F = (2^{4.9} = 29.1)</td>
</tr>
<tr>
<td>Mouse 9</td>
<td>9.8</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Mouse 10</td>
<td>9.6</td>
<td>17.8</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td><strong>10.0(^c)</strong></td>
<td><strong>14.9(^c)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Std.dev.</td>
<td>2.44</td>
<td>2.29</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Samples were normalized against the housekeeping gene 18S.

\(^b\) N.D. = not detectable.

\(^c\) Statistically significant at \(p < 0.05\).
Figure 3.4 HPSE-1 Quantification in Normal Versus Melanoma Tissues. Quantitation levels were detected by Taqman™ RT-PCR followed by \( \Delta \Delta C_T \) analysis. Mice were injected with murine melanoma cells (B16F1, B16F10, or B16B15b). Once the mice showed signs of dyspnea, they were sacrificed, and total RNA was isolated from normal (9 samples) and tumorigenic (8 samples) lung tissue. Optimized reverse transcription of RNA and amplification of specific cDNA were performed using the ABI Prism 7700 Sequence detection system (Applied Biosystems; see also “Materials and Methods”). Significant PCR fluorescent signals \( (C_T) \) for heparanase were normalized to an endogenous reference (18S). RT-PCR was performed in triplicates. Data were considered significant if \( p < 0.05 \).
Figure 3.5 HPSE-1 Immunostaining in Murine Metastatic Melanoma.
Mice (C57 BL/6) were injected with melanoma cells and subsequently lung-metastatic melanomas were extracted and formalin fixed for immunohistochemistry. Intense peroxidase staining of HPSE-1 was observed using anti-HPSE-1 PAb (see also ”Materials and Methods”). (A) Hematoxylin and eosin staining. (B) Anti-HPSE-1 PAb staining. (C) Nuclei stained by hematoxylin as a negative control. Digital images were produced on an Axioplan microscope with Advanced Spot imaging program (Diagnostic Instruments Inc., Sterling Heights, MI) with 20x objective using identical conditions for all photographs.
Figure 3.6 HPSE-1 Expression in Human Melanoma Tissues.
HPSE-1 immunohistochemical analyses of clinical specimens were performed as described in Materials and Methods using the anti-human HPSE-1 monoclonal antibody. Primary tumor (A-C) staining was negative for HPSE-1 (B) while immunostaining of lymph node tumor tissue (D-F) showed moderate positivity for heparanase (E). However, brain-metastatic melanoma tissue (G-I) showed intense staining for heparanase (H). Digital images were produced on an Axioplan microscope with Advanced Spot imaging program (Diagnostic Instruments Inc., Sterling Heights, MI) with 20x objective using identical conditions for all photographs. Hematoxylin and eosin slide (A, D, G), Anti-HPSE-1-MAb staining (B, E, H), and nuclei stained by hematoxylin as a negative control (C, F, I).
was found to aggregate around blood vessels in four out of eight brain metastatic melanoma
samples (Figure 3.7), which was not unexpected due to the role that heparanase plays in
angiogenesis. Vascularity was confirmed by staining with CD34 antibody (Kelly et al., 2003).

3.4 Discussion

In this study, we examined the relationship between the \textit{in vivo} expression and
localization of the ECM degradative enzyme heparanase (HPSE-1) and metastatic melanoma.
We provide, for the first time, evidence that 1) HPSE-1 is detected in \textit{in vivo} cases of malignant
melanoma, 2) a correlation exists between metastatic melanoma cancers and HPSE-1 expression,
and 3) elevated HPSE-1 levels are found around microvessels in melanoma specimens.

Heparanase has been shown to correlate with the metastatic potential of a variety of
tumor-derived cell lines and tissue specimens, attributable to the enzyme involvement in ECM
remodeling (Nakajima et al., 1986; Vlodavsky et al., 2001). This list includes rat mammary
adenocarcinoma, murine fibrosarcoma, human colon carcinoma and murine/human melanoma
cell lines (Toyoshima et al., 1999; Vlodavsky et al., 2001; Zetser et al., 2003). Low-metastatic
murine T-lymphoma and melanoma cells transfected with the heparanase cDNA acquire a highly
metastatic phenotype \textit{in vivo} (Wang et al., 1997). Thirdly, HPSE-1 is known to stimulate
vascular endothelial cell invasion and to induce angiogenesis in several types of cancer
(Vlodavsky et al., 2001).

Our results showed that HPSE-1 expression and localization in metastatic melanoma
cases is significantly higher than in corresponding tissues surrounding melanoma metastases or
in non-invasive, non-metastatic primary melanoma specimens. We also demonstrated its these
findings indicate that heparanase may have a central role in promoting both cell expression and
localization around blood vessels in melanoma clinical specimens. Overall, invasion and
Figure 3.7 Elevated HPSE-1 Expression Around Blood Vessels in Metastatic Melanoma.
(A) Nuclei stained by hematoxylin as a negative control. (B) Hematoxylin staining with anti-
HPSE-1 PAb showing increased HPSE-1 expression in regions around blood vessels in 4 of 8
brain metastatic melanoma samples. (C) Identification of vessels by endothelial staining with
antibody to CD34. Digital images were produced on an Axioplan microscope with Advanced
Spot imaging program (Diagnostic Instruments Inc., Sterling Heights, MI) with 20x objective
using identical conditions for all photographs.
angiogenesis are needed for the sustained growth of malignant melanoma.

Many important endogenous angiogenic factors such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and others have been reported in malignant melanoma at significantly higher levels at invasive stages of the disease during its progression to the metastatic phenotype (Bogenrieder et al., 2003). For example, VEGF overexpression can enhance growth of melanoma in the brain (Oku et al., 1998), being necessary but not sufficient for brain-metastatic growth (Yano et al., 2000). Moreover, bFGF levels in patients with metastatic melanoma are also significantly higher than those in patients with non-invasive primary melanoma (Birck et al., 1999). Finally, these factors are overexpressed in multiple malignant melanoma cell lines and in the serum of malignant melanoma patients (Ugurel et al., 2001) with most melanoma cells expressing HSPG that can act to concentrate these growth factors on their cell surface (Reiland et al., 2004). Accordingly, two considerations can be made. First, cleavage of HS within ECM and particularly within the basement membrane may be required for the migration of metastasizing melanoma cells and for remodeling of the vasculature during angiogenesis. Second, HPSE-1 can directly promote angiogenesis by releasing heparin-binding angiogenic growth factors such as bFGF and VEGF that are trapped within the ECM and/or present on the cell surface. This idea is supported by the finding that HPSE-1 increases the angiogenic response to tumors and that enhanced HPSE-1 mRNA expression correlates with tumor vascularity (Vlodavsky et al., 1999).

The mechanisms by which heparanase facilitates cancer progression likely involves more than just remodeling ECM or releasing growth factors. There is evidence that the fragments of heparan sulfate released on HS degradation maintain bioactivity and may, in fact, be more active than the native HS chains from which they are derived. In a landmark study, it was discovered
that HS fragment can either promote or inhibit the growth and metastasis of these melanoma
tumors (Liu et al., 2002b). Thus, encoded within intact HS chains are cryptic structural
elements that have the power to either positively or negatively impact the behavior of cancer
cells. HPSE-1 can be relevant because its enzymatic action produces these cryptic fragments
with resulting biological consequences. It will therefore be critical to determine the specific
function of HS fragments generated by HPSE-1 degradation in melanoma cells, and how these
fragments modulate tumor cell behavior.

As a further level of complexity, it is known that a balance between ECM degradative
enzymes and their natural inhibitors controls the overall activity of these enzymes in invasion
and metastasis. For example, demonstration that ratios of ECM-degrading matrix
metalloproteinases (MMPs) to tissue inhibitors of MMPs are important in cancer invasion and
metastasis have been reported (DeClerck et al., 1992). It is likely that HPSE-1 is also regulated
by endogenous inhibitors in normal and tumor tissues. Recently, eosinophil major basic protein
has been reported as the first identified natural heparanase-inhibiting protein (Temkin et al.,
2004). Possibly other natural HPSE-1 inhibitors can be present in melanoma cells (Keren et al.,
1989).

In conclusion, our results indicate that HPSE-1 is expressed during metastasis and
angiogenesis of melanoma cancers. Thus, HPSE-1 can be a new target molecule to inhibit
invasion, angiogenesis, and metastasis of this disease and a new prognostic factor of patients
with malignant melanoma.
4.1 Introduction

Brain-metastatic melanoma and elevated levels of heparanase (HPSE-1) are known to be strongly correlated. Elevated levels of HPSE-1 have been shown to correlate with increased aggressiveness of metastatic tumors. It is known that patients with metastatic disease show elevated levels of HPSE-1 in their sera and urine (Kelly et al., 2003; Nakajima et al., 1988), and post-operative patients with high HPSE-1 mRNA levels have a reduced chance of survival (Vlodavsky et al., 1999; Vlodavsky et al., 2001).

Malignant cells respond to paracrine and autocrine growth factor that are differentially expressed in several organs and tissues. These differential patterns of growth factor expression are a great determinant in the organ preference for metastatic colonization and growth. The primary and most well characterized paracrine factor is nerve growth factor (NGF) (Marchetti et al., 1990; Marchetti et al., 1993). NGF and NGF receptor expression are highly upregulated in melanoma progression to the highly aggressive brain metastatic-phenotype. NGF is also associated with enhancing heparanase production in brain metastatic melanoma (Marchetti et al., 2004; Walch et al., 1999).

The orthotopic brain slice model is a method where by cultures of nervous tissue can be maintained at the interface between the air and culture medium (Matsumura et al., 2000; Ohnishi et al., 1998; Stoppini et al., 1991). These cultures maintain brain cytoarchitecture such as cortical lamination and pyramidal cells. In addition, these tissues cultures retain the biochemical and electrophysiological properties of neural cells for up to one month in culture. This model makes it possible to investigate molecular events outside of the living body, which normally only occur in vivo (Matsumura et al., 2000; Ohnishi et al., 1998). The purpose our study was to use this
method to investigate the effects that both HPSE-1 and NGF have on brain metastatic melanoma cells and their invasive properties while being able to monitor the cells in in vivo-like conditions. Importantly, this brain slice model allows the ability to monitor tumor invasion at both quantitative and qualitative levels. In fact, brain-metastatic melanoma cells showed a significantly different invasion pattern depending on whether they where pre-treated with HPSE-1 or with NGF. Additionally, melanoma cells plated on these organotypic cultures showed a differential HPSE-1 expression at the protein level during time course experiments.

4.2 Materials and Methods

4.2.1 Cell Lines and Their Transfections and Cell Culture Conditions

The murine brain-metastatic cell line B16B15b was grown as a monolayer culture (Marchetti, 1993, Marchetti, 1996). Early-passage cells were stably transfected with enhanced green fluorescent protein (EGFP) DNA using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). B16B15b cells were transfected in a T25 flask, and for each tranfection 5µg of EGFP DNA and 12 µl of LipofectAMINE 2000 were incubated for 5 minutes. Following incubation, 250 µl of Opti-MEM (Invitrogen) was added and incubated for an additional 20 minutes. The EGFP, LipofectAMINE 2000, Opti-MEM solution was added to cells in addition to fresh Ham’s F-12 medium supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) (v/v) and blasticidin (10µg/ml) (Invitrogen) (1 ml). The flask was then placed in the incubator and the medium was changed within 6-24 hours. B16B15b-GFP cells were maintained as monolayer cultures in a 1:1 (v/v) mixture of Ham’s F-12 medium supplemented with 10% fetal bovine serum (FBS) (Hyclone) (v/v) and blasticidin (10 µg/ml). Cells were maintained at 37°C in a humidified 5% CO2/95% air (v/v) atmosphere and passaged using EDTA (2 mM in PBS) before reaching confluence.
4.2.2 Brain Slice Model Detecting Malignant Melanoma Cell Invasion

The brain tissue used for the organotypic brain slice models were obtained from 6-8 week old female C57BL/6 mice that were attained from Harlan Sprague Dawley (Indianapolis, IN). Prior to euthanasia they were quarantined for 1 week, and then housed in a barrier facility and fed Purine Lab Chow 5001. Mice were first anaesthetized with isoflurane and killed by decapitation. The mouse head was then plunged into 1% povidone-iodine solution. The brain was subsequently extracted from the skull and immediately submerged in ice cold sterile PBS. Next, the anterior portion of the brain was mounted and imbedded in a 3% agarose column with the anterior portion of the brain facing upward. Finally, coronal sections of the brain that were 500 µm thick were obtained with a vibratome. The brain sections were then transferred onto the polycarbonate membrane with a (0.4 µm) pore size in the upper chamber of a transwell tissue insert in a six well plate (Corning, Corning, New York), and 2 ml of DMEM/F12 medium supplemented with 10% FBS, and 100 units/ml of penicillin and streptomycin were added to the bottom well of the six well plate. After allowing the brain to equilibrate for 24 hours, B16B15b-GFP cell suspension (1x10^5 cells) was plated on the surface of the caudate nucleus and incubated for different amounts of time.

4.2.3 Brain Slice Neuronal Viability Assays

Neuronal viability in brain slices was assessed in terms of cellular uptake of propidium iodide (PI) (Sigma, St. Louis, MO) before and after the treatment with N-methyl-D-aspartic acid (NMDA) (Sigma). Morphological examinations were performed periodically on sections that had been incubated for longer than 72 hours. Brain slices were incubated with PI, after a 15 min exposure to 100 µM NMDA, for 1 hour or 24 hours. PI signals were viewed with a TRITC filter under a fluorescence microscope. PI was dissolved in a serum-free solution containing 75%
MEM, 25% phosphate buffered saline (PBS), 2 mM L-glutamine and 6.5 mg/ml glucose to a final concentration of 4.6 µg/ml as previously reported (Matsumura et al., 2000; Ohnishi et al., 1998).

### 4.2.4 Brain Slice Model Invasion Assay with Heparanase and NGF-treated Cells

Invasive properties of melanoma cells were assayed by using an orthotopic brain slice model. For invasion assays, B16B15b-GFP cells were suspended in DMEM/F12 medium supplemented with 10%FBS, and 100 units/ml of penicillin and streptomycin at a concentration of 2x10⁵ cells/µl. Directly before implanting melanoma cells on the brain slices either NGF or HPSE-1 was added to the cell suspension at a final concentration of 862 µM. Next, cells (1x10⁵ in 0.5 µl) were implanted superficially on the caudate putamen of each brain slice, three brain slice treatment groups per six well plate, and incubated for 72 hours. As a negative control, B16 B15b-GFP cells were suspended in the same medium previously mentioned and implanted on the brain slice without any treatment.

### 4.2.5 Brain Slice Model Time Course Assay

HPSE-1 expression patterns of malignant melanomas were monitored at different points of invasion through a time course assay. B16B15b-GFP cells were suspended in medium and plated as previously mentioned. Melanoma cells were implanted without any additional treatment and cells were incubated for 2, 12, and 24 hours respectively.

### 4.2.6 Tissue Preservation

After B16B15b-GFP cells and the brain slice were allowed to incubate, tissue sections were rinsed in sterile PBS and then fixed in formalin for 24 hours. Following formalin fixation, the brain slices were rinsed in sterile PBS and then incubated for 24 hours in 30% sucrose in PBS (Parlato et al., 2002). The tissue was rinsed with PBS again and flash frozen in isopentone over dry ice. The frozen sections were embedded in (Optimal Cutting Temperature medium) OCT and
mounted on a cryomold, and 10 µm thick serial sections were taken from a superior view of the 500 µm brain slices cut at -28°C (Emmert-Buck et al., 1996; Sugiyama et al., 2002).

4.2.7 Immunohistochemistry

Immunohistochemical staining was performed using a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) according to the instructions provided by the manufacturer. Briefly, formalin-fixed, OCT embedded tissue sections were mounted on poly-D-lysine coated silanized slides. Endogenous peroxidase was blocked by incubating the sections in 3.0% H₂O₂. After the blocking of nonspecific reactivity with rabbit serum for 30 min at room temperature (25°C), sections were incubated at 4°C for 60 min with the anti-rabbit polyclonal GFP anti-body raised against all variants of recombinant Aequorea GFP (Novus Biological Inc., Littleton, CO), or with anti-human HPSE-1 rabbit polyclonal anti-body raised against human heparanase (Pharmacia-Upjohn Inc., Kalamazoo, MI). Following rinsing, slides were incubated with biotinylated anti-rabbit IgG and then with Vectastain ABC reagent. Peroxidase activity was determined using NovaRED substrate kit (Vector), and the slides were counterstained with Mayer’s hematoxylin. As a negative control, sections were subjected to normal serum blocking with omission of the primary antibody.

4.2.8 Brain Slice Model Invasion Assays

Serial sections were taken from brain slices that had been implanted with treated melanoma cells or corresponding non-treated control cells. Immunohistochemistry was performed on these serial sections with anti-GFP PAb in order to definitively mark the B16B15b-GFP cells that had invaded into the brain slice tissue. Analysis of serial sections was performed blindly with a representative tissue sample. Invasion was measured in two different ways. First, invasion of melanoma cells was defined as the number of cells invading beyond the
surface of the brain per unit of area. The second method used to determine invasion was defined by measuring the depth of the most invasive cells per brain slice. The same counting area was used for each sample measurement followed by statistical analysis.

4.2.9 Statistical Analyses

All statistical analyses were conducted with the SAS® statistical package (version 9.1.2). To determine if there was a correlation between the number of cells invading and the depth of cell invasion, the Pearson correlation coefficient was calculated and significance was tested. In order to establish if this correlation could be used as a predictive model for overall cell invasiveness, regression analysis by treatment group was performed. Analysis of covariance was performed looking at heterogeneity of the slopes with cells as a covariate in order to conclude if there was a difference in invasion between treatment groups. All tests were considered significant at $P < 0.05$.

4.3 Results

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

In order to determine the effect of HPSE-1 on brain tumor invasion, B16B15b-GFP cells were treated with HPSE-1 and implanted onto an orthotopic brain slice and incubated for 72 hours. Immunohistochemistry was performed looking to visualize GFP with anti-GFP polyclonal antibody on OCT-embedded brain slice sections. Because the B16B15b-GFP melanoma cells were stably transfected, staining for GFP was a decisive way to mark invading cells with the results shown in Figure 4.1. Upon analysis of serial brain sections, HPSE-1 showed a two fold increase in the number of cells counted when compared to corresponding control groups (Figure 4.2). However, when analyzing HPSE-1 invasion as a function of cell depth into the brain tissue there was a relatively small difference between HPSE-1 treated cells
Figure 4.1 The Brain Slice Model Invasion Assay
Representative picture of invasion of tumor cells by using a brain slice model. Murine malignant melanoma cells stably transfected with green fluorescent protein B16B15b-GFP cells were plated on the caudate putamen of an organotypic brain slice as part of an invasion assay. After incubating (72 hours) the cells on the brain slices were formalin fixed, frozen, sectioned further, and IHC stained for anti-GFP as a melanoma cell marker. (A) Cells plated with no treatment, (B) cells plated after treatment with heparanase (50 ng/ml).
**Figure 4.2 Heparanase Invasion Assay Using a Brain Slice Model**
Extent of invasion into brain slices by murine brain metastatic melanoma cells as shown using quantitative analysis at 72 hours post implantation. The migration by GFP-expressing cells was measured by the number of cells invading beyond the surface of the brain per unit of area. Melanoma cells pre-treated with heparanase (50 ng/ml) had a greater number of cells invade into the brain slice per unit area than did control cells without pre-treatment. Heparanase treated cells sample (n = 75), Control group sample size (n = 100). Data were considered significant if \( p < 0.05 \).
and control cells (Figure 4.3). Statistical analysis of both the HPSE-1 treated and corresponding control groups showed a good correlation between the number of cells invaded and the depth of cell invasion. This correlation was further analyzed and determined to be a good predictive model for overall cell invasion. Analysis of covariance was performed and showed a significant difference of HPSE-1 invasiveness when compared to corresponding control groups (Table 1).

4.3.2 NFG Shows an Increase in Depth of Invasion

After treating the cell with NGF (50 ng/ml) and following their incubation (72 hours), immunohistochemistry was performed to mark the invading tumor cells in order to correctly distinguish the cancer cell from normal brain tissue. Upon analysis of the NGF invasion assay, NGF influenced invasion in a completely different way. NGF-treated samples, when analyzed for cell count, had fewer cells invade than untreated control groups (Figure 4.4). Conversely, when analyzing the depth of tumor cell invasion into the brain tissue NGF had higher depth than control groups (Figure 4.5). Statistical analysis showed a very good correlation between the number of cells invaded and depth of cell invasion for the control groups; however, the NGF treated groups had a poor correlation between cell number and depth of invasion. As a result, the NGF treated group made a poor model for cell invasiveness based on the assumption that cell number and depth correlate while the control group made for a strong predictive model. Conversely, when analysis of covariance was performed, NGF treated samples and control groups did not show a difference in invasiveness. NGF treatment groups showed a poor correlation because they had fewer cells invade, but the cells that did invade invaded further, which does not agree with the defined model.
Figure 4.3 Invasion Depth in the Presence of Heparanase Using a Brain Slice Model
Depicting the depth of invasion into brain slices by murine brain metastatic melanoma cells as shown using quantitative analysis at 72 hours post implantation. The extent of migration by GFP-expressing cells was expressed by measuring the depth of the most invasive cells per brain slice. Melanoma cells pre-treated with heparanase (50 ng/ml) showed no difference from cell without pre-treatment with regards to depth of invasion. Heparanase treated cells sample (n = 75), Control group sample size (n = 100). Data were considered significant if $p < 0.05$. 
Table 4.1 Statistical Analysis to Determine the Validity of Invasion Quantitation Methods

<table>
<thead>
<tr>
<th>Cell Treatment</th>
<th>Pearson Correlation(^1)</th>
<th>Regression Analysis(^2)</th>
<th>ANCOVA(^3)</th>
<th>Tukey Grouping(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.59430</td>
<td>0.3532</td>
<td>0.99901</td>
<td>A</td>
</tr>
<tr>
<td>Heparanase</td>
<td>0.59155</td>
<td>0.3499</td>
<td>0.86504</td>
<td>B</td>
</tr>
</tbody>
</table>

\(^1\)Pearson Correlation was to determine the correlation between the number of cells invading and the depth of cell invasion.

\(^2\)Regression analysis was performed, showing R-square values, to determine the predictive model for invasion.

\(^3\)Analysis of covariance was performed looking at heterogeneity of slopes.

\(^4\)Tukey’s grouping, designated by A and B, shows a significant difference between the treatment groups. \(p < 0.05\)
Figure 4.4 Nerve Growth Factor Invasion Assay Using a Brain Slice Model
The extent of invasion in brain slices by murine brain metastatic melanoma cells as shown using quantitative analysis at 72 hours post implantation. The extent of migration by GFP-expressing cell was measured by the number of cells invading beyond the surface of the brain per unit of area. Melanoma cells pre-treated with NGF (50 ng/ml) showed no difference in the number of cells invading than control cells without NGF pre-treatment. NGF treated cells sample (n = 90), Control group sample size (n = 68). Data were considered significant if $p < 0.05$. 
Figure 4.5 The Depth of Invasion with Invasion Assays in the presence of NGF

Invasion into brain slices by murine brain metastatic melanoma cells as shown using quantitative analysis at 72 hours post implantation. The extent of migration by GFP-expressing cells was expressed by measuring the depth of the most invasive cells per brain slice. Melanoma cells pre-treated with nerve growth (50 ng/ml) factor showed an increase in invasion when compared to cells without pre-treatment with regards to depth of invasion. NGF treated cells sample (n = 98), Control group sample size (n = 68). Data were considered significant if \( p < 0.05 \).
However, these results are correlative to previous findings who have investigated metastasis. NGF promotes tumor cell survival and aggressiveness in addition to its capabilities as a differentiation factor (Marchetti et al., 1993; Marchetti et al., 1996; Marchetti et al., 2001; Marchetti et al., 2004).

4.3.3 Melanoma Cells Show a Correlation Between HPSE-1 Expression and Incubation Time

Immunohistoshemistry was performed on brain slice model OCT-embedded sections which had B16B15b-GFP cells implanted on them and incubated for 2, 12, and 24 hours. Upon staining for HPSE-1 with an anti-HPSE-1 polyclonal antibody, we observed a low level amount of HPSE-1 staining on the B16B15b-GFP cells that had been implanted on the brain slice for only 2 hours. Additionally, the cells that were incubated for 12 hours showed a definitive increase in HPSE-1 staining and an increase in invasion. Finally, the 24 hour time point showed the heaviest staining for HPSE-1 as well as the greatest amount of invasion (Figure 4.6).

4.4 Discussion

Heparanase, which is an enzyme critically involved in degrading and remodeling ECM and basement membranes, has been shown to correlate with the metastatic potential of a variety of tumor-derived cell lines (Nakajima et al., 1983; Nakajima et al., 1984; Nakajima et al., 1988; Vlodavsky et al., 2001). Upon plating HPSE-1 treated brain-metastatic melanoma cells on brain slices, we were expecting a large number of cells to invade very aggressively. However, the HPSE-1 invasion assay had a high number of cells invade, but the cells didn’t invade deep into the tissue. In fact, there was little difference between the HPSE-1-treated cells and the control cells as to depth of invasion. This may be due to the fact that HPSE-1 degraded the basement membrane of the brain slice, but did not actually stimulate the cells to a
Figure 4.6 HPSE-1 Time Dependent Expression in Brain-Metastatic Melanoma Cells. B16B15b-GFP cells were implanted on the brain slices and at 2 hours. (A,D), 12 hours. (B,E), and 24 hours respectively. (C, F) post-implantation the experiments were stopped and IHC was performed. (A, E, F) Nuclei stained by hematoxylin with an isotyped matched mouse monoclonal antibody used as the primary antibody for a negative control for IHC staining. (A, B, C) Hematoxylin staining with anti-HPSE-1 PAb showing increased HPSE-1 expression at increasing time points. Digital images were produced on an Axioplan microscope with Advanced Spot imaging program (Diagnostic Instruments Inc., Sterling Heights, MI) with 20x objective using identical conditions for all photographs.
more aggressive metastatic state. The result from this would be the cells migrating into the area of the brain that the HPSE-1 degraded away, but the cells were not aggressive enough to actively invade farther into the tissue.

The NGF receptor (p75\textsuperscript{NTR}) is abundantly expressed in many melanoma tumors, and p75\textsuperscript{NTR} is especially over-expressed during the later stages of melanoma progression (Marchetti, 1993). It is known that malignant melanoma develops from cells undergoing an uncontrolled cell survival, growth, and proliferation to a neoplastic setting. This response is dependent upon these cells having an enhanced sensitivity to either autocrine or paracrine growth factors (Nicolson, 1993). Through responsiveness to these growth factors, melanoma cells can become clonally dominant within a tumor. This is what makes the paracrine factor NGF such an interesting molecule because it has the ability to influence cancer cells to progress into malignant aggressiveness and survivability. However, for NGF to be able to promote this effect, there has to be a high concentration of the p75\textsuperscript{NTR} present on the cell surface. It is this p75\textsuperscript{NTR} that makes the results of our invasion assay from the brain slice model of interest. NGF treated cells had a lower number of cells invading over time; however, cells that invaded migrated to a greater depth. This could mean that only a few of the cells overexpressed p75\textsuperscript{NTR}. Consequently, when the NGF was added to our brain slice models only a few of the cells became clonally dominant, but the few that invaded did so aggressively (Marchetti et al., 1993; Marchetti et al., 2004; Walch et al., 1999).

The orthotopic brain slice model can present an \textit{in vitro} model of the central nervous system in which neuronal architecture can be maintained. This model would create a co-culture between the melanoma cells and the brain tissue environment that allows a more realistic growth environment yet still being rather easy to manipulate treatment. First, it provides the ability to
analyze processes at the cell and molecular level while the cells are growing in a viable
environment. Second, it allows the opportunity to run an invasion assay without having to add
chemoattractants to the assay (Stoppini et al., 1991). Third, having the brain slice model for brain
metastatic melanoma is especially helpful because it is thought that metastasis is dependent on
certain host tissue properties, particularly the host microenvironment. For these reasons, this
model is of considerable use in the future research of brain metastatic melanoma. This model has
been previously used in studying mechanisms of invasion in glioma cells and astrocytoma cells
(Jung et al., 2001; Matzner et al., 1992; Ohnishi et al., 1998; Yoshida et al., 2002). This novel
model has shown that brain derived neurotrophic factor mediates activity-dependent dendritic
growth in neocortical interneurons in developing organotypic cultures (Jin et al., 2003). The
othotopic brain slice model has proven useful in both the study of physiological and
pathophysiological developmental processes.
CHAPTER 5: SUMMARY AND CONCLUSIONS

The first part of our studies was focused on *in vivo* experimentation and in looking at HPSE-1 expression from tumors that metastasized to the lung. Since metastatic melanoma cells express high levels of HSPG, and their tumors grow highly vascularized, we analyzed melanoma tissue specimens for HPSE-1 expression from experimental animals as well as from patients. The results from these analyses demonstrated that HPSE-1 mRNA is significantly elevated in metastatic melanoma when compared to adjacent lung tissue. We showed that there were increased levels of HPSE-1 protein expression in metastatic melanoma clinical specimens while primary tumors result in low level HPSE-1 protein expression. Another finding was that the presence of HPSE-1 appeared to be more concentrated around blood vessels and vascular regions in brain-metastatic melanoma specimens, a relevant finding since heparanase in known to be involved in angiogenesis.

Since we had already shown that HPSE-1 expression is upregulated in tumor tissue when compared to normal corresponding tissue, we wanted to prove mechanistically that HPSE-1 promotes invasion in *in vivo*-like conditions. Therefore, the objective of the second part of this project was to establish an organotypic culture system that can be of considerable usefulness in studying the process of brain-metastatic melanoma invasion. First, this model provides a better representation of the ECM molecules normally encountered by invading melanoma cells. Second, it gives the ability to monitor tumor invasion at both quantitative and qualitative levels. In fact, brain-metastatic melanoma cells showed a significant increase in invasion when treated with HPSE-1 versus untreated cells. Additionally, melanoma cells plated on these organotypic cultures showed differential expression of HPSE-1 at the protein level during a time-course experiment.
Finally, this model is important because it can be used to determine mechanisms in cancer invasion and metastasis. First, it can be used to determine *in vivo* biological effects in conjunction with approaches that selectively block the expression of molecules implicated in cellular invasion. Furthermore, with this model, biological effects are quantifiable, hence it can be used to determine therapeutic efficiency.


APPENDIX: LETTER OF PERMISSION

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Professor D. A. Spandidos.

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To: "Brian Murry" <bmurry1@lsu.edu>
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Subject: RE: Approval to use copyrighted material
Date: Mon, 13 Jun 2005 09:24:23 +0300

Yes, you can.

Yours sincerely,

D.A. Spandidos

---------------------------------------------------------------
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Professor D.A. Spandidos,

I am currently writing my thesis for my graduate degree program, and I the first author of a manuscript that has been published in the International Journal of Oncology. **Selective heparanase localization in malignant melanoma.** 2005 Feb;26(2):345-52. I know that you have legal ownership of the paper, but I wanted to know if I could use the paper in a part of the thesis. If this is possible please let me know the steps to follow. Thank you for your assistance in this matter.

Sincerely,

Brian P. Murry

Louisiana State University
VITA

Mr. Brian Murry graduated from Louisiana State University (LSU), Baton Rouge, Louisiana, in May of 2002, receiving a Bachelor of Science with a major in microbiology and a minor chemistry. He worked in Dr. Dario Marchetti’s cancer tumor biology laboratory at the LSU School of Veterinary Medicine for a year. After which, he enrolled in the LSU Veterinary Medical Sciences degree program through the Department of Comparative Biomedical Sciences in September of 2003 to begin his experimental studies in cell and molecular biology of brain metastatic melanoma.