A Study of the Impact of Membrane Organization of Glycosphingolipid E-selectin Ligands and Glycoproteins on Head and Neck Cancer Cell Adhesion to Vascular Endothelium

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## This thesis titled

A Study of the Impact of Membrane Organization of Glycosphingolipid E-selectin Ligands and Glycoproteins on Head and Neck Cancer Cell Adhesion to Vascular Endothelium

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#### Abstract

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A previous study by the Burdick lab showed that head and neck squamous cell carcinoma (HNSCC) cells bind to vascular endothelium through an adhesive interaction between oncologic glycosphingolipids and endothelial E-selectin. It was also suggested that the GSL ligand activity may be inhibited due to steric hindrance by neighboring proteins as evidenced by a major increase in adhesion when HNSCC (JHU-013-SCC) cells were treated with a protease. This thesis project investigates that inhibitory interaction and analyzes the impact of lipid raft association of GSLs and nearby proteins on adhesion inhibition. Parallel plate flow chamber analysis showed that lipid raft disruption, as well as lentiviral silencing of CD44, induced statistically significant increases in tethering of JHU-013-SCC cells to E-selectin. It was shown that lipid raft disruption had a greater effect than CD44-silencing. CD44-silencing exhibited an effect on binding almost synonymous to protease treatment suggesting that CD44 is the sole protein contributor to the inhibition. In addition, it was shown that CD44 present on the surface of JHU-013-SCC cells is localized within the lipid raft microdomains, therefore increasing the efficacy of the inhibition due to its probable association with nearby Eselectin ligands. Overall, this study showed that CD44, found in lipid raft microdomains, is a major contributor to protein inhibition of GSL ligand-mediated adhesion of HNSCC cells to endothelial E-selectin.

Approved: \_\_\_\_\_

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#### **Chapter 1. Overview of Cancer Characteristics and Metastasis**

Cancer is believed to originate from a tumor-initiating somatic cell that gains enough mutations genetically or epigenetically to evade standard regulatory processes of the body. Two of the main types of genetic alterations are loss of function in tumor suppressor genes and gain of function in oncogenes. In a review describing cancer hallmarks, Hanahan and Weinberg introduced the idea that all cancer types share specific traits:

"self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis," (Hanahan & Weinberg, 2000).

Cancer is known to affect almost every area of the body. Head and neck squamous cell carcinoma (HNSCC) is the sixth most prevalent cancer in the world, most often caused by excessive use of tobacco or alcohol; however, some strains of human papillomavirus (HPV) have been shown to cause tumors, especially in the oral and oropharyngeal tissues. HNSCC accounted for 6% of the total malignancies in 2010 in the United States (American Cancer Society, 2010; Leemans et al., 2011). Due to advances in modern medicine, most cancers are very treatable if found during early stages of growth. However, once a cancer metastasizes, the outlook is not as encouraging: 90percent of cancer fatalities occur due to metastasis. HNSCC most often metastasizes to the lymph nodes and lungs, and the five year survival rate of HNSCC decreases from 83% to 32% once the cancer has metastasized (American Cancer Society, 2010). Therefore, insights into the mechanisms of metastasis are needed in order to develop strategies to prevent and treat late-stage cancers.

Currently, the molecular mechanisms driving cancer metastasis are unknown. Nevertheless, it is commonly believed that metastasis follows a similar pattern as that of leukocyte recruitment to sites of inflammation (Talmadge & Fidler, 2010). Once a tumor cell breaks off from the primary site and migrates through the basement membrane of its host tissue, it enters the blood stream and is classified as a circulating tumor cell (CTC). The CTC proceeds through the vascular system, and due to a combination of vascular compressive and shear forces acting on the cancer cell in the blood stream, the CTC may physically interact with the vascular endothelium initiating a multistep adhesion cascade. Through interactions between adhesion molecules present on both the CTC and the endothelium, the CTC tethers to the endothelium and rolls until it becomes firmly adherent. Once firmly adherent, the CTC is able to cross the vascular endothelium into an underlying tissue with the help of specific recruitment chemokines. The CTC is now free to invade the tissue, proliferate, and form a metastatic colony (Talmadge & Fidler, 2010). Identifying the molecules involved in this adhesion cascade is vital to understanding the overall metastatic process.

## **Chapter 2. Cancer Adhesion Molecules**

## 2.1 The Selectin Family

Previous research has identified an important group of type I transmembrane proteins, called selectins, that play an important role in cell adhesion (Barthel, 2007). Lselectin is expressed on leukocytes and is a vital component of leukocyte homing to lymph nodes and sites of inflammation. P-selectin is expressed on platelets and endothelial cells following inflammation. E-selectin is expressed on the surface of endothelial cells after stimulation with inflammatory cytokines, including interleukin-1 $\beta$ (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Barthel, 2007). All three selectins are comprised of a lectin domain, an epidermal growth factor region, a section of short consensus repeats, a transmembrane domain, and a small cytoplasmic domain. The three molecules differ only in the number of short consensus repeats: L-selectin has 2 repeat sections, E-selectin has 6, while P-selectin has 9 (Vestweber & Blanks, 1999). The importance of selectin molecules was identified when their role in leukocyte homing and neutrophil recruitment was discovered. L-selectin was discovered through the development of an antibody that bound to high endothelial venules, sites of leukocyte homing. E-selectin was first identified during the development of an antibody that bound to activated endothelium and prevented the adhesion of neutrophils. P-selectin was first found as a transmembrane molecule held within platelet granules then later found to be expressed on activated endothelium. Once P-selectin was discovered to be similar to the other two selectins, P-selectin was identified as having a major role in neutrophil adhesion to platelets and endothelium (Vestweber & Blanks, 1999).

While the role of the selectins has been well characterized for immune responses, a considerable amount of research of the contribution of the molecules, particularly Eselectin, in tumor metastasis has been performed in the last decade. E-, L-, and P-selectin have been shown to mediate the adhesion of colon, prostate, and breast cancers, in addition to multiple leukemic cell lines, to vascular endothelium or neutrophils that subsequently adhere to the endothelium, a process necessary for metastasis (Barthel, 2007; Dimitroff, 2004; Mannori, 1995; Nimrichter, 2008; Shirure, 2011). We recently showed that HNSCC binding to cytokine-activated vascular endothelium is mediated solely by E-selectin under flow conditions (Wood et al., manuscript in progress). The vascular drainage routes from the sites of the body commonly affected by HNSCC pass through the pulmonary cavity. Läubli and Borsig showed that many lung metastases were present in conjunction with upregulation of E-selectin is lung vasculature (Läubli & Borsig, 2010a). The combination of the vascular routes accessible to circulating HNSCC and upregulated adhesion molecules of these areas may contribute to the high occurrence of lung metastases of HNSCC.

#### 2.2 Sialyl Lewis X and Sialyl Lewis A

Because of its terminal lectin domain, E-selectin primarily binds ligands with specific carbohydrate moieties. The first of these moieties to be characterized were sialyl Lewis X (sLeX) and sialyl Lewis A (sLeA) (Barthel, 2007). sLeX and sLeA have been demonstrated to be pivotal in mediating vascular adhesion of colon and prostate cancer cells (Izumi et al., 1995; Takada et al., 1993). Currently, there is ongoing research to

validate the use of a sLeA mAb (CA19-9) and a mAb recognizing carcinoembryonic antigen (CEA) as a prognostic tool for colorectal cancer (Basbug et al., 2011). And while sLeX expression is not currently used as a prognostic tool, there is much evidence to support a trend between increased sLeX expression and metastatic potential (Izumi et al.1995; Numahata et al., 2002). sLeX was discovered to be upregulated on the surface of the HNSCCs using both *in vitro* and *in vivo* analysis techniques (Farmer et al, 1998, Wenzel et al., 1995). In addition, sLeX was shown to mediate static binding of HNSCC cells to vascular endothelium, which suggests a role for this molecule in HNSCC metastasis (Wenzel et al., 1995).

## 2.3 E-selectin Ligands

sLeX and sLeA residues are most often bound to a macromolecule backbone. These backbones have been shown to be either lipids or proteins. Because of this, a great amount of research has been dedicated to discovering both glycoproteins and glycolipids on the surface of cancer cells that mediate adhesion to the selectins. As a result of advances in proteomic technology, the majority of the E-selectin ligands discovered have been glycoproteins. Perhaps one of the most important ligand discoveries was that of Pselectin glycoprotein ligand-1 (PSGL-1) (Moore et al., 1995). PSGL-1, along with a variety of additional glycoprotein ligands including E-selectin ligand-1 (ESL-1), and the CD44 glycoform HCELL, facilitate the vascular adhesion of prostate and colon cancer cells (Burdick et al., 2006; Dimitroff et al., 2005; Hanley et al., 2005). While the expression of these and many other glycoprotein ligands has been extensively characterized, the knowledge of oncologic glycolipid ligands pales in comparison. Yet, past research has identified glycolipids possessing properties that meet the requirements for selectin ligands. Through static and dynamic adhesion assays, glycosphingolipids (GSLs) were shown to preferentially bind to E-selectin rather than L- or P-selectin (Alon et al., 1995; Burdick et al., 2001; Burdick et al., 2003; Vogel et al., 1998). This, in combination with the presence sLeX-decorated glycolipids on metastatic colon carcinoma cell colonies, suggests a primary role of GSLs in mediating cancer metastasis (Matsushita et al., 1991). Similarly, our previous work shows HNSCC adhesion was protease-resistant (Wood et al., manuscript in progress); however, binding was eradicated when cells were treated with a sialidase prior to perfusion (Wood et al., manuscript in progress). In addition, GSLs isolated from HNSCCs expressed E-selectin reactivity and mediated binding to endothelial cells in both the planar and cellular orientations (Wood et al., manuscript in progress). Overall, these studies prove that GSLs contribute to the adhesion of multiple cancer types. However, the identity of these lipid ligands remains unknown.

## **Chapter 3. Lipid Raft Microdomains**

In addition to initiating the adhesion cascade, the binding of cancer cells to vascular endothelium plays an important role in transducing signals that allow for the progression of the adhesion cascade (Laubli & Borsig, 2010b). Because the plasma membrane is a fluid bilayer of lipids and proteins, the signaling molecules, including Eselectin ligands, are generally confined to lipid raft microdomains in order to be in the optimal proximity to propagate the signal. Lipid rafts are rigid sections of the cell membrane enriched in cholesterol and ceramide, which inhibit the diffusion of enclosed molecules (Alberts et al., 2008; Patra, 2007). Glycosphingolipids are known to cluster and associate in lipid raft microdomains, though the cause is currently unknown. GSLs and their clustering abilities were first studied using artificial membranes and micelle structures. Through a series of experiments, it was shown that GSLs can associate in established domains, and they possess the ability to create unique domains made of only GSLs (Prinetti et al., 2009). Proteins, e.g. kinases, are also known to associate within lipid raft domains. It is believed that transmembrane proteins may localize within lipid rafts through interactions with GSLs or glycophosphatidylinositol (GPI)-linked proteins. This interaction can bring said proteins into contact with other raft components or can possibly segregate the proteins from other molecules excluded from the rafts.

The most well studied role of lipid rafts on cellular behavior is the initiation and progression of signaling pathways. The importance of membrane organization for tumor cell adhesion was shown in a 2006 study by Huang et al. where treatment of cancer cells with emodin, a naturally-occurring resin that disrupts lipid raft structures, was shown to

decrease the cholesterol and sphingolipids present on the cellular membrane (Huang et al., 2006). This caused a decrease in binding of the cancer cells to extracellular matrix (ECM) components. The decrease in adhesion was attributed to the disruption of the interaction between focal adhesion kinase and integrin ligands. It was shown that the emodin treatment had no effect on integrin expression, and therefore the decrease in adhesion must be due to signal transduction disruption (Huang et al., 2006). In addition, disruption of lipid raft domains was shown to decrease the chemotaxis of breast cancer cells. When breast cancer cells were treated with methyl- $\beta$ -cyclodextrin (M $\beta$ CD), a cyclic oligosaccharide which sequesters cholesterol, the frequency of ligand-induced adhesion, which precedes chemotaxis, decreased. It is believed that the obstruction of GSL E-selectin ligands and the implications of interactions with nearby proteins may help to explain the intricate details of the behavior of membrane GSLs on HNSCC cells.

#### **Chapter 4. CD44 and Cancer Cell Adhesion**

In addition to demonstrating GSL-mediated adhesion to vascular endothelium, our previous work differed from other studies in that HNSCCs expressed no significant levels of E-selectin reactive membrane proteins (Wood et al., manuscript in progress). In all other cancer models, GSLs are thought to have a redundant adhesive nature, adding to the contribution attributed to glycoprotein ligands (Burdick et al., 2003). Due to a significant increase in binding when treated with a protease, we believe that GSLs are the preferential E-selectin ligands on HNSCCs, while proteins play an inhibitory role, blocking the adhesion of the lipids due to steric hindrance.

Based on ongoing work in the Burdick lab studying the effect of hyaluronic acid on adhesion, it is believed that CD44 is the main protein contributing to the inhibition of GSL-mediated adhesion. CD44 is a transmembrane protein that exists in multiple forms due to alternative splicing. Certain variants, specifically CD44v6, have been found to be upregulated in many cancers, including HNSCC (Cichy & Puré, 2003; Orian-Rousseau, 2010). CD44 is most notable for its high adhesion affinity for hyaluronic acid (HA), which was shown by Aruffo et al. (1990). When cells were treated with hyaluronidase, all CD44-mediated binding was eliminated (Aruffo et al., 1990).

The interaction between CD44 and HA is important for the induction of metastatic invasion and inhibition of apoptosis. More specifically, it has been shown that when HA is not internalized, a process dependent on its ligation to CD44, apoptosis is induced in breast cancer cells (Thankamony & Knudson, 2006). The presence of the HA-CD44 interaction was also shown to be important in colon carcinoma invasion into

matrigel (Orian-Rosseau, 2010). Furthermore, it has been determined that CD44 must be associated within lipid raft structures in order to internalize HA. In a study by Thankamony and Knudson (2006), epithelial cells transfected to express wild type CD44 were incubated in MBCD and tested for HA adhesion and internalization. While the disruption of the lipid rafts did not cause a significant decrease in HA binding to CD44, no internalization of HA was observed (Thankamony & Knudson, 2006). In conclusion, CD44 is known to play an important role in cell adhesion and lipid raft mediated processes, but its role as an inhibitory molecule of adhesion has not been investigated. While intensive work has been done in identifying the role of lipid raft components in signaling pathways, the interactions and impact of the components on each other from a cell adhesion standpoint has yet to be studied. This is an important area of research because it could create a link between initial attachment of cancer cells to endothelium and the rest of the hypothesized cascade of metastasis by identifying the molecules necessary for tethering and rolling, which may interact with signal cascades that allow for firm adhesion and diapedesis. The greater the amount of information that is known about cancer metastasis, the better the potential for development of targeted therapeutics.

## **Chapter 5. Specific Aims**

**Hypothesis:** Steric hindrance attributed to CD44 found in the membrane lipid rafts impedes the glycosphingolipid-mediated tethering ability of JHU-013-SCC cells to the vascular endothelium (Figure 1).



**Figure 1. Hypothesized surface organization model of JHU-013-SCC cell.** In order to test the above hypothesis, the following specific aims were investigated experimentally.

Specific Aim 1: Determine whether CD44 removal increases tethering ability of HNSCC binding to E-selectin. This aim tested the hypothesis of the CD44 steric interference on binding in a series of experiments in which CD44 was removed from the surface of the HNSCCs and the effects on adhesion to CHO-E cells was observed. CD44 was removed by protease treatment and shRNA silencing of the CD44 gene. The parallel plate flow chamber was used to determine the effects on binding of removal of CD44. The adhesion of the treated or silenced cells was compared to untreated and vector control cell lines. Specific Aim 2: Characterize the organization of the glycosphingolipids and CD44 in the lipid rafts and what implications the organization has on the adhesion ability of HNSCC. This aim analyzed the hypothesis that CD44 and the GSL E-selectin ligands are colocalized within lipid raft microdomains by disrupting the raft structures while keeping the cells intact or extracting the lipid rafts. Lipid rafts were extracted from the HNSCCs and probed for CD44 through dot blot analysis. To see the lipid raft effects on binding, the cells were treated with M $\beta$ CD to disrupt the lipid rafts and analyzed using parallel plate flow chamber. Both untreated control and CD44-silenced cells were used for the lipid raft extraction and their individual results were compared in order to deduce the effect of the gene silencing. The same was done for the flow adhesion assays.

## **Chapter 6. Materials and Methods**

## 6.1 Sterile Cell Culture

The head and neck cancer cell line JHU-013-SCC (neck node metastasis) was a generous gift from Dr. Vicente Resto (University of Texas- Medical Branch, Galveston, TX). HL60 (human leukemic) cells were purchased from the American Type Culture Collection. JHU-013-SCC and HL60 cells were cultured in RPMI supplemented with 10% fetal bovine serum (FBS) and 1X penicillin-streptomycin (Pen-Strep). The Chinese Hamster Ovary cells transfected to express E-selectin (CHO-E) cell line was a generous gift from Dr. Robert Sackstein (Harvard Medical School, Boston, MA) and were cultured using MEM supplemented with 10% FBS, 1X Pen-Strep, 1X non-essential amino acids, and 1 mM sodium pyruvate. Primary human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Walkersville, MD). These cells were cultured in M-199 supplemented with 10% FBS, 1X Pen-Strep, 1X L-glutamine, 0.025 g/mL heparin, 0.0125 g/mL endothelial mitogen; final solution was sterile filtered. All cells were grown in an incubator kept at 37°C and 5% CO<sub>2</sub>.

#### 6.2 Antibodies and Reagents

Monoclonal antibodies HECA-452, CD62E (68-5H11), sLeX (CSLEX-1), CD44 (515), all isotype controls (mIgG1, mIgG2a, mIgM, rIgM) and streptavidin-PE conjugated secondary antibody were purchased from BD Biosciences (San Jose, CA). The remaining conjugated secondary antibodies (mIgG-, mIgM-, rIgM-FITC; mIgG-, hIgG-AP) were purchased from Southern Biotech (Birmingham, AL). An additional

monoclonal antibody used (CD44 (2C5)) and recombinant murine E-selectin/human Fcchimera were purchased from R&D Systems (Minneapolis, MN). Biotinylated hyaluronic acid-binding protein and sLeA antibody (KM231) were purchased from Calbiochem (La Jolla, CA). Cholera toxin-B-tagged Alexa Fluor 488 and streptavidin- tagged Alexa Fluor 488 tagged secondary reagent were purchased from Invitrogen (Carlsbad, CA).

Neuraminidase ( $\alpha$ -2,3,4,6,8 sialidase, *V. cholera*) was from Roche (Indianapolis, IN). Bromelain (protease from pineapple stem) and methyl- $\beta$ -cyclodextrin (m $\beta$ CD) were obtained from Sigma Aldrich (St. Louis, MO). Interleukin-1 $\beta$  (IL-1 $\beta$ ) was purchased from R&D Systems.

## 6.3 Lentiviral Gene Silencing

JHU-013-SCC cells were seeded into a multi-well plate at 30-percent confluence. Following 24-hours of growth incubation, the growth media was replaced with media containing 8 µg/mL polybrene and Mission shRNA lentiviral construct TRCN0000057563 targeting CD44 or vector control (Sigma-Aldrich) at the desired MOI. The cells were allowed to incubate for 24 hours, and the media was replaced with fresh growth media. After an additional day of growth, the cells were treated with 2 µg/mL puromycin for drug selection. Puromycin was used because the lentiviral shRNA constructs carry a puromycin-resistance gene. Therefore, when puromycin is added to the media, only the cells that were successfully transduced will survive. The cells were then cultured normally in media containing puromycin until positive gene silencing was established. The degree of gene silencing was evaluated by flow cytometry. Specificity of silencing was confirmed by the lack of effect on E-cadherin, another membrane protein.

## 6.4 Flow Cytometry

Flow cytometric analysis was performed as previously reported (Burdick et al, 2006). Freshly harvested cells were resuspended in 0.1% bovine albumin serum (BSA)/Dulbecco's phosphate buffered saline (DPBS), aliquoted into individual wells of a 96-well plate, and centrifuged; supernatant was removed. Primary antibody solution was added to each well and allowed to incubate for 30 minutes on ice. After incubation, the cells underwent multiple wash cycles before being resuspended in the appropriate fluorescent-tagged secondary antibody solution. The plate was left on ice for 30 minutes in the dark. Following additional washes, the cells were transferred to FACS tubes and analyzed using the FACSAria Special Order Research Product flow cytometer (BD Biosciences).

## 6.5 Polar Lipid Isolation

Polar lipids were extracted from HNSCC cells according to the method of Svennerholm (Schnaar, 1994). Three-times the volume of ice-cold distilled water was added to one volume of frozen cell pellets, and the pellets were homogenized on ice. The aqueous cell mixture was added to glass tubes of rapidly stirring methanol at room temperature. Chloroform was added to make the final concentration 4:8:3 (CMW). The CMW mixture was allowed to stir for one hour. The mixture was then centrifuged. The supernatant was removed and placed in a new centrifuge tube. The solids were reextracted by resuspending them in CMW (4:8:3), centrifuged, and the supernatant was added to the previous supernatant. Water was added to the supernatant solution to bring the CMW concentration to 4:8:5.6. The tube was centrifuged, and the upper organic layer was removed and added to a fresh centrifuge tube. CMW (4:8:5.6) with 10 mM KCl in place of pure distilled water was added to the bottom layer; the tube was centrifuged. Upper layers were combined. The final organic solution was twice subjected to reverse phase chromatography through a C18 Sep-Pak column (Waters Corporation, Milford, MA) to collect the lipids from the bulk liquid. The polar lipid extracts (PLE) were then eluted with methanol and dried.

## 6.6 Thin Layer Chromatography/Immunostain Overlay

PLE (see above) were spotted on 5x5 cm silica gel plates (Merck, Darmstadt, Germany), leaving a one centimeter border on each side. When fully dry, the plates were placed into a glass chamber containing a CMW (60:35:8) developing solution. The plates were removed once the liquid had reached 1 cm from the top of the plate. The plates were dried and prepared for further processing.

In order to visualize the lipids, the dry plate was sprayed with a heat reactive solution of concentrated hydrochloric acid, 2% aqueous resorcinol, and 5 mM cupric sulfate (1:2:0.01) solution, covered with a glass plate, and heated at 120°C for 20 minutes. Sialylated lipids appeared blue, while non-sialylated species were brown.

To test the lipid species for E-selectin reactivity, the plates were immunostained using Eselectin/Fc chimera and alkaline-phophatase secondary antibodies and reagents. First, the dry plates were dipped in hexanes, followed by 0.1% PIBM in hexanes. The plate was then blocked for 30 minutes in 0.1% BSA/0.05% Tween-20/tris balanced saline (TBS). After washing, the plate was treated with the chimera for 1 hour at room temperature. The plate was subsequently washed and placed in secondary antibody under the same incubation conditions. The plate was then washed thoroughly washed with TBS and developed using Promega Western Blue (Promega, Madison, WI). The reactive species appeared dark over a light background. Specificity of staining was determined by comparing stained profile with that of lipids pre-treated with 0.1 U/mL neuraminidase for 30 minutes.

## 6.7 Membrane Lipid Raft Isolation

Membrane raft fractions were isolated from JHU-013-SCC cells using a method previously described by George, Wu, & Wu (2010). Cells were grown to 80-90% confluence on 10 cm plates, washed, and treated with 1.25 mM 3,3'-Dithiobis[sulfosuccinimidylpropionate] (DTSSP) (Thermo Scientific, Waltham, MA) and incubated for 1 hour on ice to crosslink the lipids and proteins. Cells were then washed, scraped using PBS and 5 mM EDTA, pelleted, and stored at -80°C overnight. Once thawed and washed with cold TBS, the cells were lysed using 0.1% Triton-X membrane raft isolation buffer containing protease inhibitor. Subsequently, cells were forced through 23-gauge needle twenty times. The solution was centrifuged at 1000 rpm at 4°C and the supernatant was removed and transferred to a new tube. Optiprep (Axis-Shield PoC AS, Oslo, Norway) solution was added to the supernatant at various concentrations to create an iodixanol gradient. The tube was then spun in Beckman Coulter Optima L90 ultracentrifuge for 5 hours at 31,300 rpm at 4°C. The resulting fractions were collected and analyzed.

## 6.8 Lipid Raft Dot Blot Analysis

Lipid raft fractions were analyzed for molecular expression using dot blot analysis as previously reported (Elyassaki & Wu, 2006). 10 µL of each lipid raft fraction was loaded into the BioDot apparatus (Bio-Rad Laboratories) along with positive control. JHU-013-SCC whole cell lysate was used as positive control for CD44 staining. The samples were then vacuum loaded into a pre-wet nitrocellulose membrane. The membrane was allowed to dry for 30 minutes and was then blocked in FBS for 4 hours. The membrane was washed and incubated in primary antibody solution overnight at 4°C. The membrane was washed and incubated in the appropriate alkaline phosphatase (AP)conjugated secondary antibody solution for two hours at room temperature. After washing, the membrane was developed in AP colorimetric or chemiluminescent reagent.

## 6.9 Parallel Plate Flow Chamber

Cancer cells suspended at 10<sup>6</sup> cells/mL in 0.1% BSA/DPBS were added to a syringe connected by plastic tubing to the parallel plate flow chamber. The aqueous cell solution was pulled through the gasket by an automated syringe pump set at a volumetric

flow rate corresponding to 1 dyne/cm<sup>2</sup> wall shear stress. The flow chamber sat atop an inverted microscope. A camera attached to the microscope projected the flow through the channel to a television set where the visual output was recorded using a VCR. The tethering events of the cancer cells to plated IL-1 $\beta$ -stimulated HUVECs were counted and compared. Enzymatic treatments were applied to cells before perfusion to compare adhesion effects of various treatments. To cleave proteins, the cells were treated with 0.1% (w/v) bromelain for 30 minutes at 37°C. In order to disrupt the membrane raft structures, cells were treated with methyl- $\beta$ -cyclodextrin (0.1 g/mL) for 10 minutes at 37°C immediately before perfusion.

## 6.10 Statistical Analysis

Data are expressed as average  $\pm$  SEM. Statistical significance was determined using two-way ANOVA with a subsequent t-test (Bonferroni) or one-way ANOVA with a subsequent t-test (Bonferroni). Data with probabilities less than 0.05 were considered to be significant.

## **Chapter 7. Experimental Results**

## 7.1 JHU-013-SCC cells express known cell adhesion molecules

JHU-013-SCC cells were tested by flow cytometry for known surface markers that have been shown to play a role in cell adhesion to vascular endothelium and specifically, E-selectin. These included CD44 and its receptor hyaluronic acid (HA), as well as sialofucosylated molecules, sLeX, sLeA, HECA-452, and VIM-2. The HECA-452 antibody recognizes a variety of sialofucosylated decorations, including sLeX, sLeA, and cutaneous lymphocyte antigen (CLA); the VIM-2 recognizes a sialofucosylated structure with internal rather than terminal fucosylation, which is not probed by the HECA-452 mAb and was therefore tested independently. Figure 2 shows the results from flow cytometry analysis. The JHU-013-SCC cells were shown to be positively expressive of all six structures. The unfilled lines represent the isotype control, while the dark stained region is the specific monoclonal antibody.



Fluorescence Intensity

**Figure 2. JHU-013-SCC cells express known cell adhesion epitopes.** JHU-013-SCC cells were probed for a variety of known selectin ligand epitopes via flow cytometry. The cells showed positive reactivity for four sialofucosylated mAbs (HECA-452, sLeX, sLeA, and VIM-2), as well as for CD44 and HA. Isotype control is represented by the unfilled lines, while specific antibody expression is shown using the filled in regions. Graphs shown are representative of n=3.

## 7.2 JHU-013-SCC cells express glycosphingolipid E-selectin ligands

In order to determine if the lipid-mediated E-selectin adhesion was possible, the polar lipids were extracted from the JHU-013-SCC cells and subjected to analysis using thin layer chromatography. Thin layer chromatography separates the individual lipid species on a silica-coated glass plate based on their polarity due to migration induced by an organic solvent. The lower polarity lipids migrate at a faster rate and therefore are shown at higher regions on the plate. HL60 cells were also subjected to polar lipid

extracted and loaded on the plates in order to serve as a positive control (Stroud, YEAR). The resolved plates were tested using two techniques. The first consisted of spraying the plate in a hydrochloric acid (HCl): resorcinol: cupric sulfate (CuSO<sub>4</sub>) reagent that detects the presence of both sialylated and non-sialyated lipids when heated. This profile is shown in the left panel of Figure 3. As seen in the profile, the majority of JHU-013-SCC lipids fall into the mono-sialylated range, while di- and tri-sialylated species are also present. In the second treatment, the plate was overlayed with recombinant murine Eselectin/F<sub>c</sub> chimera and the appropriate secondary antibody ( $\alpha$ -human-IgG-AP). When treated with Western Blue, the reactive species were visible. This profile is shown in the middle panel of Figure 3. The dark stained lines represent the species that most likely contribute to the E-selectin adhesive nature of the JHU-013-SCC cell lines. They mainly range in the mono, di-, and tri-sialylated region, rather than the asialo-region. This is consistent with past work that has shown that sialylation is required for E-selectin adhesion (Laübli & Borsig, 2010). Specificity of staining was confirmed by comparing the E-selectin stained lipids with lipids that were treated with neuraminidase to cleave the sialic acid groups prior to loading. The neuraminidase lipid plate was also stained with Eselectin/F<sub>c</sub> chimera, and the bands present on both E-selectin-stained plates were taken to be due to non-specific staining (Figure 3).



**Figure 3. JHU-013-SCC cells express E-selectin-reactive glycosphingolipids.** GSLs from HL60 and JHU-013-SCC cells were extracted and loaded onto silica gel plates. The plates were developed in an organic solvent mixture and either sprayed with resorcinol to detect all lipid species, or fixed and stained with E-selectin/Fc chimera to detect E-selectin reactive lipids. Specificity of E-selectin staining was determined by running neuraminidase-treated lipids on an additional plate. Dark bands on middle pane represent E-selectin-reactive glycosphingolipid species. Data are representative of n=3 independent experiments.

7.3 Protease and lipid disruption treatments altered surface expression of JHU-013-SCC

cells in a predictable manner.

In order to determine the effect of membrane organization on JHU-013-SCC cell adhesion to vascular endothelium, two treatments were applied to the cells before perfusion in the chamber device. Cells were either treated with bromelain, a widely acting protease, or methyl-β-cyclodextrin, which sequesters cholesterol consequently disrupting all membrane raft structures. It was determined by flow cytometry that bromelain treatment successfully cleaved proteins, including CD44, from the surface of the JHU-013-SCC cells. On the other hand, when treated with m $\beta$ CD, the surface expression of the JHU-013-SCC cells remains at a similar level, as expected since m $\beta$ CD only affects the surface amount of cholesterol. Neither treatment had a major impact on HECA-452 reactivity levels.



Fluorescence Intensity

Figure 4. CD44 and HECA-452 expression levels after protease and m $\beta$ CD treatments. When JHU-013-SCC cells were treated with bromelain, the proteins, such as CD44, the main protein of interest, were cleaved from the surface of the cells. The HECA-452 expression did not change with bromelain treatment. When JHU-013-SCC cells were treated with methyl- $\beta$ -cyclodextrin to disrupt lipid raft microdomains (with or without subsequent bromelain treatment), cell surface expression of CD44 and HECA-452 epitopes remained relatively similar to untreated control. Isotype control is represented by the unfilled lines, while specific antibody expression is shown using the filled in regions. Histograms shown are representative of n=3.

7.4 The E-selectin dependent tethering of JHU-013-SCC cells to activated endothelium

## increases with lipid raft disruption

Because of past research that showed that glycosphingolipids and CD44 are

known to associate in lipid raft structures (Patra, 2007; Prinetti et al, 2009; Thankamony

& Knudson, 2006), the effect of lipid raft disruption on E-selectin-mediated adhesion was tested using the parallel plate flow chamber. As seen in Figure 4, when JHU-013-SCC cells were treated with methyl- $\beta$ -cyclodextrin (m $\beta$ CD) to disrupt membrane rafts the number of tethering events of the tumor cells to IL-1 $\beta$  stimulated human umbilical vein endothelial cells (HUVECs) increased significantly. JHU-013-SCC cells had a 3-fold increase in tethering ability when treated with mBCD to disrupt lipid rafts and about a 2fold increase when treated with bromelain (Figure 4). Combining the two treatments had no additive effect to the tumor cells' tethering ability relative to m $\beta$ CD treatment alone (Figure 4). Each treatment was repeated using HUVECs treated with an  $\alpha$ -CD62E monoclonal antibody (mAb) that blocked binding to E-selectin. As seen in Figure 4, blocking E-selectin on the surface of the HUVECs eradicated all binding, therefore all tethering was determined to be E-selectin dependent. Overall, the data show that integrity of membrane lipid raft structures affects successful adhesion of JHU-013-SCC cells to vascular endothelium. This effect may be due to the lipid rafts restricting the diffusion of the GSL ligands. Therefore, once the lipid rafts are removed, the GSLs can diffuse freely in the membrane, removing themselves from any nearby proteins or other molecules that are inhibiting their adhesion. On the other hand, it is possible that the lipid rafts play a role in the progression of a signaling pathway that is disrupted when the lipid rafts are removed, and allows for greater adhesion of the GSLs to E-selectin.



Figure 5. Disruption of lipid rafts and protease-treatment of JHU-013-SCC cells increases E-selectin dependent adhesion to IL-1 $\beta$ -activated HUVECs. JHU-013-SCC cell tethering to IL-1 $\beta$ -activated endothelium was observed using a parallel plate flow chamber. Cells were perfused at rate so that the wall shear stress was 1 dyn/cm<sup>2</sup>. The difference in tethering ability between enzyme-treated cells and untreated cells was determined. Lipid raft disruption, as well as protease treatment, was shown to significantly increase the tethering ability of the tumor cells. Additionally, a combination treatment of m $\beta$ CD and bromelain did not significantly affect the tethering ability of the JHU-013-SCC cells with respect to pure m $\beta$ CD treatment alone. The adhesion was found to be E-selectin dependent in all cases as treatment with a monoclonal antibody that blocked the binding site of E-selectin led to eradication of all tethering events. Data are average  $\pm$  SEM of n=3, \*p<0.05 as determined by two-way ANOVA post-hoc with a ttest (Boferroni) relative to untreated JHU-013-SCC cells over untreated HUVEC cells, #p<0.05 as determined by one-way ANOVA relative to untreated HUVEC cells over untreated HUVEC cells.

## 7.5 Lentiviral silencing of CD44 increases adhesion of JHU-013-SCC cells to E-selectin

In order to determine whether CD44 is responsible for the inhibitory effect on

JHU-013-SCC cell adhesion to E-selectin, cells were treated with lentiviral particles to

silence CD44. The success of the lentiviral transduction was determined by flow

cytomtery analysis. The mean fluorescence intensity (MFI) of CD44 expression

decreased by 84±18% when JHU-013-SCC cell were silenced with lentiviral particles compared to untreated JHU-013-SCC cells (Figure 6).



Fluorescence Intensity

Figure 6. Lentiviral transduction decreased CD44 expression on surface of JHU-013-SCC cells. JHU-013-SCC cells transduced with lentiviral particles were analyzed by flow cytometry and compared to untreated as well as vector control cells. As seen in CD44 expression histograms, lentiviral silencing greatly decreased the surface expression of CD44 on JHU-013-SCC cells. Isotype control is represented by the unfilled lines, while specific antibody expression is shown using the filled in regions. Data are representative of n=5.

CD44-silenced cells, as well as positive control cells, were analyzed using the flow chamber apparatus. Each cell line was treated with mβCD and/or bromelain and perfused through the flow chamber over a CHO-E monolayer; tethering events were observed, recorded, and compared. As seen in Figure 7, CD44-silenced cells bound to CHO-Es at a significantly higher rate than both untreated and vector control cells. Additionally, it was shown that the combination of either treatment with CD44-silencing had no further effect on tethering ability (Figure 7). Moreover, CD44-silencing increased tethering to a similar degree as bromelain indicating that CD44 may well be the only protein contributing to the inhibition of E-selectin adhesion. Overall these data show that the main inhibitory effect on the GSL-mediated adhesion to E-selectin is due to CD44. These results indicate that CD44 is a major contributor to the inhibition of JHU-013-SCC cells to E-selectin. This inhibition may be due to physical shielding of the GSLs due to the large steric area of CD44, or perhaps due to its participation in some sort of signaling pathway made possible by the colocalization of CD44 and the GSL E-selectin ligands.



Figure 7. CD44-silencing increases adhesion potential of JHU-013-SCC cells to Eselectin. The adhesion potential of lentiviral CD44-silenced JHU-013-SCC cells (CD44shRNA) was compared to untreated cells and vector control cells. Cells were perfused at rate so that the wall shear stress was 1 dyn/cm<sup>2</sup>. CD44-silencing of cells significant increased the adhesion potential of JHU-013-SCC cells. However, CD44 did not have an additive effect on adhesion potential when combined with other treatments (m $\beta$ CD and/or bromelain). Data are average ± SEM of n=5; \*p<0.05 as determined by two-way ANOVA with a t-test (Boferroni) relative to untreated JHU-013-SCC cells, #p<0.05 as determined by one-way ANOVA relative to untreated JHU-013-SCC cells.

# 7.6 Glycolipid E-selectin ligand profile of JHU-013-SCC cells remains unchanged by CD44-silencing

## 7.6.1 Determination of Equivalent Loading

In order to ensure CD44 silencing had no effect on glycolipid ligand expression of JHU-013-SCC cells, it was first necessary to establish equivalent loading parameters for the untreated, vector control, and CD44-silenced PLE samples. This was obligatory because profiles would be compared for both band location as well as degree of staining. It was shown through flow cytometry that CD44-silencing led to no statistical significant change in MFI of GM1, a known sialylated ganglioside, by adhesion to its ligand cholera toxin B (Figure 8A). PLEs were therefore spotted onto a TLC plate and stained using cholera toxin B conjugate. Using imaging software, the intensity of the bands was quantified. The quantities were normalized based on the intensity of the untreated PLE. These ratios were then used to equalize the loading in the E-selectin comparison TLC (Figure 8B).

## 7.6.2 Effect of CD44 silencing on glycolipid E-selectin ligand profile

In order to determine whether silencing CD44 had an effect on the expression of glycolipid E-selectin ligands present on the JHU-013-SCC cells, PLEs from untreated, vector control, and CD44-silenced cells were loaded onto a TLC plate, developed, and stained with recombinant murine E-selectin/Fc chimera. Specificity was confirmed by

treating the PLEs with neuraminidase, which cleaves all terminal sialic acid structures, prior to loading and comparing the staining to the untreated lipid plate. There was no distinct change in the staining profiles between the CD44-silenced cell PLE and either control (Figure 8B). Therefore, CD44-silencing has no effect on the glycolipid E-selectin ligand expression of JHU-013-SCC cells.



**Figure 8. Lentiviral silencing of CD44 does not change E-selectin reactive lipid profile of JHU-013-SCC cells.** (A) JHU-013-SCC cells were stained for GM1 using flow cytometry. All JHU-013-SCC cells (untreated, vector control, CD44-silenced) positively express GM1, and the relative expression of GM1 was not altered with CD44silencing. (B) PLE from JHU-013-SCC CD44-silenced cells was run on a TLC plate in parallel with untreated and vector control PLEs and stained for E-selectin reactivity. Nonspecific staining was determined based on neuraminidase treatment of lipids prior to loading. No significant change was seen in the stained profiles of the CD44-silenced cells as compared to either control. All results are representative of n=3.

## 7.7 CD44 is located within lipid raft microdomains

In order to determine whether or not CD44 and the GSL E-selectin ligands are colocalized within lipid rafts, the lipid rafts were extracted from JHU-013-SCC cells using an OptiPrep gradient. The resulting fractions were loaded with the appropriate positive controls onto a nitrocellulose membrane and probed with a CD44 monoclonal antibody. CD44 staining was evident in the lipid raft fraction (Fraction 2; Figure 9A). The specificity of binding was determined by comparing the membranes to those stained with the corresponding isotype control primary antibodies. Overall, this experiment showed the majority of membrane CD44 is contained within lipid raft microdomains.



Figure 9. CD44-reactivity found in fraction 2 of lipid raft isolation fractions. Lipid rafts were extracted via an OptiPrep gradient and ultracentrifugation. Each fraction was loaded onto a membrane and stained with  $\alpha$ -CD44 monoclonal antibody. Colorimetric development showed fractions that contained CD44. CD44 was only found in the second lipid raft fraction, the same fraction that contains the lipid rafts. Data are representative of n=2.

## **Chapter 8. Discussion**

In a previous study by the Burdick lab, it was suggested that membrane proteins may inhibit glycosphingolipid-mediated adhesion of head and neck squamous cell carcinoma cells to E-selectin present on vascular endothelium (Wood et al., manuscript in progress). Therefore, this study analyzed the effect of membrane organization on glycosphingolipids-mediated adhesion and associated proteins of JHU-013-SCC cells to E-selectin. Through a combination of lipid raft disruption and protease treatments, it was determined by parallel plate flow chamber adhesion assays that membrane raft structures create an inhibitory effect on adhesion. When these raft structures were removed from the cells, there was a major increase in adhesion events. In addition, protease treatment showed similar results though the protease treatments had less of an impact on adhesion than lipid raft disruption. All together, these results suggested that the GSL E-selectin ligands are shielded by neighboring protein structures that are most likely highly glycosylated and confined to the lipid raft structures. This inhibition may be due to steric association of proteins physically shielding the ligands preventing their adhesion, limitation of membrane fluidity by the lipid raft structures preventing the GSL ligands from diffusing out from under the proteins, or it could be related to a signaling pathway involved in adhesion.

In other studies performed on lipid raft disruption of cancer cells, disrupting the lipid raft structures decreased the amount of adhesion due to interruption of signaling pathways (Huang et al, 2006; Liu et al, 2007). Because in this study lipid raft disruption increases adhesion, the signaling pathway involved may be inhibitory. Alternatively, it is possible that the negative impact on adhesion by lipid raft disruption is downstream. For example, this study only looked at the impact on tethering, the first step of the adhesion cascade. It is possible that lipid raft disruption interrupts a signaling pathway that may facilitate later steps such as rolling and therefore, there may be an increase in tethering events, but fewer cells may reach the later steps or complete the adhesion cascade.

In the majority of studies performed on E-selectin ligands, proteins were found to be the main ligands present on cancer cells. However, this study along with the previous study by the Burdick lab (Wood et al., manuscript in progress), showed that in HNSCC cells proteins most likely inhibit rather than facilitate E-selectin adhesion. CD44 has been found by past studies to be a very important in preventing apoptosis of breast cancer (Thankamony & Knudson, 2007). In addition, it was noted that organization in lipid raft structures was also important for the adhesion process (Thankamony & Knudson, 2006). In line with these conclusions, in this study, it was determined that CD44 was the main protein contributing to the inhibition of the GSL adhesion. This was proven through parallel plate flow chamber using CD44-silenced cells. When JHU-013-SCC cells were silenced, adhesion increased approximately the same amount as when the tumor cells were treated with a protease. In addition, treating CD44-silenced cells with a protease did not further increase the impact on the adhesion to E-selectin. CD44-silenced cells were also treated with m $\beta$ CD to disrupt the lipid rafts; the overall impact of this combination treatment on adhesion was similar to that of normal JHU-013-SCC cells treated with mβCD. This is most likely due to the fact that when the lipid rafts are disrupted the GSL E-selectin ligands are able to diffuse out from under CD44 and become available to bind

to E-selectin. Therefore, the inhibition of CD44 is eliminated and removing the CD44 molecule does not provide any additional advantage. It is also possible that the inhibition may be related to a signaling pathway and when the lipid rafts are disrupted the majority of the inhibition is removed.

Glycosphingolipids and lipid rafts are vital for a multitude of signaling pathways including those involved in the immune response (Alberts et al., 2008) and a variety of other cellular functions (Patra, 2007). At this point only a few studies have been performed on the impact of lipid rafts on cancer cell adhesion (Huang et al, 2006; Liu et al, 2007). However, in order to fully understand the entire mechanism of cancer metastasis, the pathways involved must be identified. This study has opened up the opportunity to study the involvement of lipid rafts not only in the promotion of adhesion, but also in the inhibition, which could help model possible therapeutics to prevent the progression towards metastasis.

#### **Chapter 9. Future Aims**

## 9.1 Discover the identity of specific glycosphingolipids E-selectin ligands

In this study, as well as previous studies by the Burdick lab, it has been shown that glycosphingolipids are the major E-selectin ligands present on HNSCC cells. However, the identity of the individual glycosphingolipids species and their relative contribution to the adhesion ability of the cells is currently unknown. In the future, the glycosphingolipids species could be purified by high performance liquid chromatography (HPLC). The HPLC products could then be analyzed using mass spectrometry to reveal the structure and identity of each GSL species.

To analyze the relative contribution of each GSL species to the overall adhesion ability of the cell, a combination of far eastern blotting (Taki & Ishikawa, 1997) and the blot rolling assay (Burdick et al, 2006) could be utilized. Lipids would be loaded onto a TLC plate, then transferred to a PVDF membrane using a hot iron. CHO-E cells would then be perfused over the membrane. The areas where the CHO-E cells adhered would represent the GSL species responsible for the adhesion interaction. In addition, the purified HPLC products could be adsorbed to polystyrene microspheres and perfused over a CHO-E monolayer. This would assess the adhesion potential of each lipid species in a pseudo-cellular orientation.

## 9.2 Comparative analysis of binding kinetics

Binding kinetics analysis can glean a significant amount of information pertaining to the exact nature of the adhesion interactions between cancer cells and vascular endothelium. While this study explored the surface organization of the molecules involved in the E-selectin adhesion, it did not investigate the kinetics of the interactions. In order to calculate the kinetics parameters, such as association and dissociation constants and binding affinity, the adhesion forces would be measured using optical tweezers (Sun et al, 2009). The HNSCC cell would be captured in the optical trap and brought into contact with the CHO-E layer. The HNSCC cell would then be slowly moved away from the monolayer, and the force of attachment/detachment would be measured (Long, Lü, & Sun, 2006). This process could be repeated for all treatments used in this study to gain a better understanding of why adhesion increased.

## 9.3 Investigating lipid raft signaling pathway

Lipid rafts have been shown to play an important role in cell signaling pathways (Patra, 2007), including those involved in cancer cell adhesion and chemotaxis (Huang et al, 2006; Liu et al, 2007). This study showed that the lipid raft orientation of the GSL E-selectin ligands affected the adhesion potential of HNSCC cells. Additionally, past work has shown that E-selectin ligand adhesion is a pivotal event in adhesion in that it initiates the necessary signaling pathways for cancer cell rolling (Läubli & Borsig, 2010b). It is possible that the adhesion of JHU-013-SCC cells to E-selectin increases with lipid raft disruption because the disruption interferes with a signaling pathway. Identifying the specific signaling pathway that is initiated by E-selectin adhesion, such as SYK and Src kinase pathway (Läubli & Borsig, 2010b) may elucidate the intricacies involved in the HNSCC metastatic process. It may be possible to identify inhibitory/promotional

signaling pathways involved in cancer metastasis, pathways that may extend to a variety of cancer types.

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