Distinct Glycosylations Lead to Breast Cancer Cell Adhesion to E-selectin

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ABSTRACT

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The majority of deaths caused by breast cancer are due to cancer metastasis. Breast cancer cells express glycoprotein E-selectin ligands that can be recognized and bind to E-selectin expressed by vascular endothelium. The purpose of this study is to define the type(s) of glycosylation(s) leading to E-selectin ligand function of cell surface proteins expressed by breast cancer cells. BT-20 cells were treated with deoxymannojirimycin (DMJ) to prevent N-linked glycosylation of glycoproteins, whereas cells were treated with benzyl-N-acetyl-α-galactosaminide (Bzl-GalNAc) to prevent O-linked glycosylation. Immunoprecipitation, western blot staining with various antibodies, and flow cytometry were used to measure glycosylation influence on E-selectin ligand function[1]. Real-time PCR showed glycosylation inhibitors treatment did not significantly affect BT-20 CD44 expression. Breast cancer cells E-selectin ligand function is affected by N-linked glycosylation of glycoproteins. A mathematical model also was developed to describe BT-20 cell adhesion to E-selectin. These findings will lead to better understanding of cancer metastasis.

DEDICATION

To my beloved family

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1: INTRODUCTION

1.1 Epidemiology, diagnosis and treatments of cancer

Apoptosis is programmed cell death that should occur in cells. Absence of apoptosis can lead to abnormal proliferation of cells. This abnormal proliferation of cells can be benign or malignant. In the latter case, the tumor becomes malignant and is considered to be a condition called cancer[2]. In other cases such as leukemia, there is no real formation of cell masses but causes the same effect as other cancer and spread to different parts of the body through the process of metastasis. Cancer in a particular system or tissue metastasizes to different organs, causing systemic damage of the cells. In reality, the formation of cancer causes different effects on people[3]. It can essentially form in any organ or tissue. Any element of the human body is susceptible to developing cancer[4], and there are more than 100 different types of cancers. This is a systemic failure that is caused by numerous factors in both human body and environment. For the formation of cancer, there are many factors that have been identified as carcinogenic. These carcinogenic factors include such as heredity, lifestyle, infections and environment.

Millions of people in America have suffered from cancer. According to the American Cancer Society (ACS), 1,685,210 new cancer cases will be diagnosed in 2016. And it has been estimated that 595,690 Americans are expected to die of cancer. This roughly translates to cancer detection in 1,630 people per day. According to the governmental documentation reports, the 5-year relative survival rate for all cancers diagnosed between 2005 to 2011 has been on the rise to 69%, from 49% from the years

1975 to 1977[5]. This is an encouraging statistic. Nevertheless, certain forms of cancer cannot be easily resolved. It depends on the type of cancer and the stage at which it has been diagnosed. To evaluate the stage of cancer clinicians use TNM guidelines. In this TNM system, the clinics use a differential staging system. It is the extent of primary tumor (T), analysis of the lymph nodes (N), and the presence of the distant metastasis (M). From this, the stages of cancer have been identified as 0, I, II, III, IV. Some kinds of cancer use different systems to analyze its stages. For the case of breast cancer, these TNM guidelines are used to detect the levels of progression and the stages of the cancer[6].

One-third of women and half of men in the US have already developed or will develop cancer in their lifetime. Cancer cells may get into bloodstream or lymph vessels to spread to other parts of body and form new tumors, which is called cancer metastasis. 90% of deaths that are caused by cancer are a result of cancer metastasis[7]. Mammographic screening is used for primary breast cancer diagnosis[2]. In addition, cancer can be diagnosed by observing cells or tissue samples from patients under a microscope. Nowadays diagnosis and prognosis of cancer have significantly improved. Treatments, for example surgery, radiation therapy, chemotherapy and other methods, have been used on cancer patients. Progress has also been made toward personalized therapy. Tests of DNA, RNA and proteins of sample cells can help in future diagnosis.

1.2 Characteristics of breast cancer and breast cancer metastasis

Breast cancer is caused by mutations related to issues in apoptosis, leading to abnormal proliferation of the cells. Gender (being a woman) and age (growing older) are

two major risk factors for breast cancer[8]. Some of the risk factors that cause cancer are caused by the activities indulged in by some people. The term “risk factor” is used to denote some of the activities that have the possibility of causing breast cancer. It is a relative term that is used to explain in detail the possibilities that could cause breast cancer. Some of the risk factors are weight gain after the age of 18, usage of menopausal hormone therapy, being physically inactive, increased levels of alcohol consumption, and cigarette smoking, especially after the first pregnancy (women). Some studies state that disruptive sleep patterns could also contribute to the formation of breast cancer[9]. In some cases, certain factors are not modifiable; those are the familial history, increase in age, and inherited mutation of BRCA1 and BRCA2 or other genes that are vulnerable to develop cancerous conditions[10]. Health conditions such as high bone mineral, osteoporosis and high levels of sex hormones have been attributed to causing breast cancer. In America, according to statistical observations, about one of eight women will have invasive breast cancer during their lifetime. Breast cancer is the most common cancer in American women next to skin cancer. It is estimated by the American Cancer Society that about 40,450 women will die because of breast cancer in 2016[11]. Men can also develop breast cancer.

Metastatic breast cancer is still considered incurable, leading to the majority of breast cancer deaths[12]. Breast cancer metastasis is when breast cancer cells spread to other parts of the body from its primary site through bloodstream or lymph vessels. It is a multi-step cascade that begins when cancer cells separate from the primary mass, intravasate through capillary walls, spread through the bloodstream, and finally seed new

colonies in distant organs. Any failure in any of these steps will lead to suspension of metastasis[13]. Simultaneously, cancer cells need to escape the host's immune response for the purpose of survival. Detection of circulating tumor cells (CTCs) is a rising way to predict breast cancer metastasis. Circulating tumor cells are tumor cells that circulate in bloodstream originating from primary site[14]. CTCs are important for cancer metastasis research. With increased understanding of breast cancer metastatic cascade, cell-surface receptors expressed on cancer cell and their cognate ligands involved in metastasis will lead to critical improvements in the diagnosis and prognosis of breast cancer.

The most evident reason to analyze and study breast cancer is to prevent the high mortality rates. For this, there is a need to understand the dynamics of metastasis, which has been identified as one of the most important facets of these diseases. Breast cancer that starts out as a local disease is found to metastasize to the lymph nodes and distant organs. At a primary level diagnosis, prognostic markers are used to analyze the impact of these systemic diseases. In this, metastatic capacity is an important parameter to understand the impact of the tumor. Some research has pointed towards the direction that breast cancer metastasis is acquired late through the tumor genesis events. Some have stated that this is a systemic disease. Nevertheless, any form of development of tumor in the breast cancer needs to be analyzed.

There are currently three processes followed to identify breast cancer, namely, the screening test, diagnostic test and monitoring tests. Screening tests such as clinical breast exams are given to the general public to identity if they are at risk for developing cancer. It is not an accurate method, but it is an initial screening test that is used to detect any

abnormality or any cell mass. A clinical breast exam is suggested at least every 3 years for women in their 20s and 30s. Annual mammographic screening is advised for women in their 40s and older. Accurate determination of breast cancer and confirmation is done through analysis of the abnormal cell growth in breast. For diagnostics, a range of tests is prescribed to detect the presence of cancer. One of the most accurate tests is the use of biopsy. It has been found that early diagnosis of the condition leads to easier rectification of the issues. Apart from this, blood cell count, biomarkers, bone markers, blood chemistry, blood magnetic resonance imaging (MRI), chest x-rays, ductal lavage, fluorescence in situ hybridization (FISH) tests, mammogram, molecular breast imaging, and ultrasounds are also used as diagnostic tests. Thermography is one of the other diagnostic tests that are used to identify and monitor the progression of cancer.

Women who have developed breast cancer have higher rates of mortality when they are identified at a later stage. The survival rate of people once they have developed cancer is directly related to the stage at which it has been identified. Stage II breast cancer survival rate is as high as 93%. But the survival rate for stage IV is 22% as shown in Table 1[6]. The reason for the high mortality rates is due to metastasis of cancer cells. This also shows the imperative need for identifying breast cancer at a much earlier stage. After providing treatment, monitoring tests are used to watch for progression of the disease. If the cancer develops again or relapses, the monitoring tests can be used to identify the progression of the cancer.

Table 1. Breast cancer TNM tumor classification system and survival rate[6].

Stages	TNM	**Survival Rate**
Stage 0	Tis, N0, M0	Close to 100%
Stage I	T0 or T1, N0 or N1 mi, M0	Close to 100%
Stage II	Any T, N0 or N1 (but not N1mi), M0	93%
Stage III	Any T, Any N, M0	72%
Stage IV	Any T, Any N, M1	22%

Newer molecular technologies such as DNA microarrays have helped in developing a prognosis for the disease and aids in understanding of the metastasis process[2]. Apart from these proven technologies to determine cancer progression, a number of new investigations have been undertaken. For instance, a number of novel treatments and methods of diagnosis have been derived to understand breast cancer mechanism in adults. It has been found that studying the impact of HER-2 (Human Epidermal growth Factor type 2) is an effective way to diagnose the disease[15]. For breast cancer, the most important defense mechanism is early diagnosis no matter what method is used.

For the purpose of identifying the cancer, selectins play a potentially important role in detection. Selectins are a family of cell adhesion molecules (CAM). All the selectins are found are single chain transmembrane proteins that are primarily glycoproteins. Selectins primarily are found to bind to sugar moieties, and they are actually cell adhesion proteins that primarily bind to sugar polymers. There are three

types of selectin that have been identified: E-selectin, P-selectin and L- selectin. E-selectin is found on blood vessel endothelial cells; L-selectin is found on leukocytes; P-selectin is found on platelets and endothelial cells. Selectins in general are found to influence how cells bind and play an important role in the dynamics of cell adhesion. It has been observed that the selectins are found to play an important role in cancer cell metastasis [16, 17]. The mechanism in which E-selectin and E-selectin ligands play an important role in breast cancer metastatic cascade has been elucidated in detail in the following section.

1.3 Roles of E-selectin and E-selectin ligands in breast cancer metastatic cascade

It has been found that a considerable portion of breast carcinoma knowledge has been derived from the cell lines derived from breast cancer. They aid in providing an unlimited source for analyzing cancer cells. BT-20 is one of the oldest forms of cell lines that have been developed. It was developed in 1950s[18]. In actuality, it was difficult to develop the exact process that emulates carcinoma. BT-20 is one of the most utilized breast cell lines and has aided in gaining extensive knowledge about carcinoma functions [19]. Subsequent to this discovery, MCF-7 was developed in the year 1973. It is more sensitive towards hormone sensitivity and is considered to be an ideal model to study the hormone response of the cells. MCF-7 and another line, MDA-MB-231, are known for their prominent function of the adenosine receptor types[20]. MCF-7 does not have adenosine receptors owing to the lack of Ca^{2+} levels. Adenosine-mediated responses in MDA-MB-231 cell lines were observed to nonselective agonist[21]. A2B receptor in

cancer cells serves as a target to promulgate cell growth and proliferation[22]. This shows that the cell lines can be used to understand the mechanism of breast cell carcinoma.

The process of cancer cell adhesion to endothelium during metastasis entails a cascade of events known as tethering, rolling, adhesion and extravasation. In the first step, the circulating cancer cells tether to the endothelium. This is partially because of the cancer cells interaction with the erythrocytes known as the marginalization effect. This leads to the cancer cells being concentrated towards the vessel wall. The first initial contact between cancer cell and endothelial cell is because of the mechanical properties. After the process of initial tethering, the cancer cells move in the path of blood flow in the system. It successfully detaches itself from endothelial cells then immediately into the second stage, which is the rolling process. The rolling behavior is uniquely owing to the velocity that is normally 10 to 100 fold less than cancer cells exhibiting non-adherence characteristics[8]. In this process, there is a need to factor in the rolling velocity. Once rolling is initiated, the activated cancer cells migrate across the endothelium to the extravascular space, or the extravasation step. For all these processes, there is a need for the cancer cells to have successful cell molecular interaction. Hence, a combination of molecular interaction and cancer cells' mechanical nature that is similar to the mechanism leukocytes used for adhesion and extravasation through endothelium are a prerequisite for this action to be initiated[23]. These selectins involved in cell molecular interaction are transmembrane glycoproteins that are present in the endothelial structure and form transient bonds with selectin ligands on cancer cells. Based on the binding specificity, E-selectin ligands are expressed by breast cancer cells. They are found in

several breast cancer cell lines. E-selectin plays an important role in adhesion of breast cancer cells to endothelial cells by converting rolling to firm adhesion[8, 24]. To further understand the dynamics of this behavior, biophysics can be used to understand the nuances.

Endothelial moieties are required for adhesion of the molecules. It has been found that the ligand-receptor bonds are non covalent in nature. The aggregate force of the adhesion between the cancer cells and endothelium is able to counter the external pressure exerted by the cancer cells owing to the blood flow. Ultimately, it is the relationship between these two aspects that determine the strength of the specific cancer cell adhesion to the endothelial cell[8]. Cancer cell adhesion to endothelial cells is controlled by the vascular selections that are found to be complementary to the carbohydrate ligands. This is an integral aspect in the initial steps of the cell adhesion cascade. For instance, L-selectin, E-selectin, and P-selectin bind to their ligand P-selectin glycoprotein ligand-1 (PSGL-1) and sialyl-Lewis^x like carbohydrates. PSGL-1is a glycoprotein that is found on leukocytes and prominently binds with P-selectin. PSGL-1 is also found to bind with L-selectin and E- selectin. There is a need to understand the molecular measurement and elasticity of these compounds to further understand the dynamics of the situation. This process of PSGL-1 binding to the selectin has been widely studied and mathematical models have been build to better understand this process[25]. This process of PSGL-1 binding to the selectins is found to be an imperative process for the initial tethering and rolling mechanism. Size, force and torque of the cells are important determinants to understand the effect of shear force. For the purpose of

understanding the nuances of rolling, a 3D computational model that encompasses the Monte Carlo method was developed. It was found from this analysis that hydrodynamic and receptor-ligand bond forces explain the ligand adhesion in detail[26]. The Arrhenius equation has been used as a basis to develop successful models to describe the nuances of the cell adhesion[2, 26]. Alternatively, studying the actual impact and the biophysical aspect of these PSGL-1 measurements can also be performed by measuring changes in thermal fluctuations by microscopic cantilever. While E-selectin binding to other E-selectin ligands has similar processes, it lacks a mathematic model. From all the studies that have been done on PSGL-1 binding to selectins, we can develop similar mathematic models for E-selectin ligands, specifically CD44 in breast cancer cells, binding to E-selectin.

Blood flow applies disruptive hydrodynamic force on breast cancer cells while there is an adhesive force between breast cancer cell selectin ligands and vascular endothelial cell selectins. The competition of these two forces controls the overall adhesion process. At the cancer cell accumulation step, circulating cancer cells tether to endothelial cells and then respond to hydrodynamic drag force by rolling. The contact adhesive area is quickly translated along the vessel wall during rolling process. The force (F) and torque (T) exerted by blood flow during this process is related to local hydrodynamic environment (as shown in Figure 1). The bonds formed between receptors and ligands because of adhesive force could be catch bonds or catch-slip bonds. Lifetime of catch bonds increases by tensile mechanical force. Catch bonds are stronger at high shear rate than low shear rate. When shear rate is lower than the shear threshold, breast

cancer cells can roll along on endothelial cells as bonds formed between receptors and ligands form and break rapidly. Lifetime of slip bonds is shortened with increasing force. Catch-slip bonds are bonds in which lifetime increases with increasing shear at first, and consequently decrease in lifetime when a shear threshold is reached. E-selectin can form catch bonds or catch-slip bonds with its ligands[27].

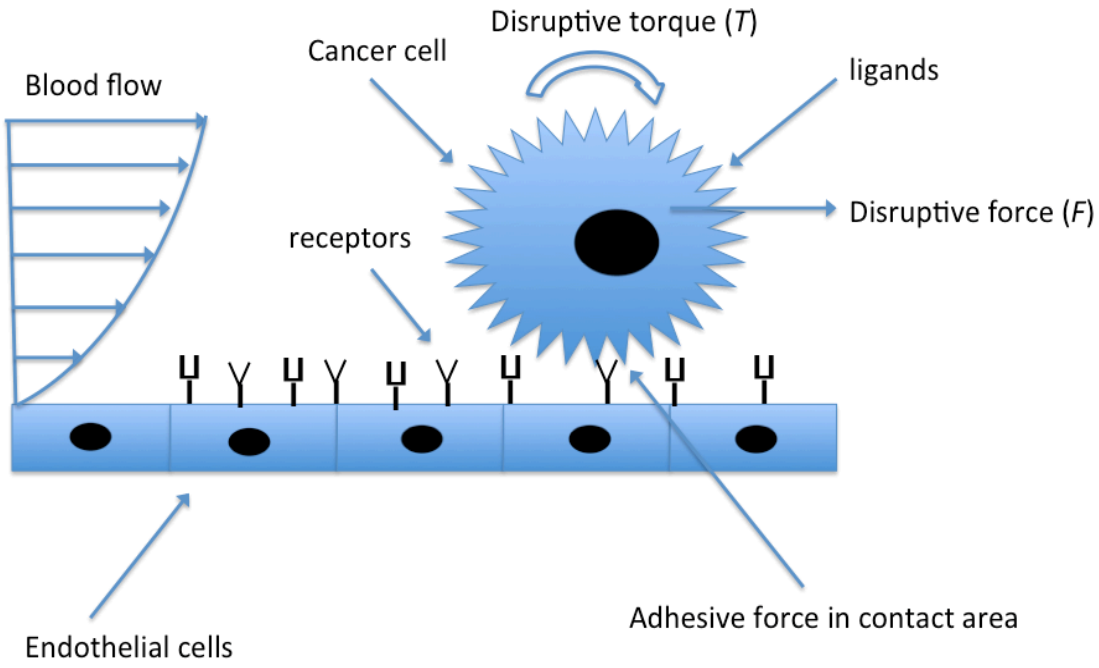

Figure 1. Force analysis during the cancer cell metastasis process. There is competition between the adhesive force and disruptive force.

CD44, a type of cell-surface glycoprotein and E-selectin ligand, plays a critical role in cell-cell adhesion, cell interactions and cell migration[11]. Among the 12 most common isoforms of CD44, the 4 isoforms that are mainly expressed in breast carcinoma cells are CD44s, CD44v3, CD44v4, CD44v6[28]. It has been found that the alternative

splicing is the reason for many differential isoforms[11], in which inserting various forms of exons into the extracellular membrane's proximal region generates the variant isoforms. For this purpose, force spectroscopy is used to analyze the biophysical and molecular behavior. CD44 also binds to hyaluronan and fibrin (ogen). CD44 binding to hyaluronan and fibrin (ogen) is analyzed by combining force spectroscopy with biochemical interventions[29]. This provides a partial explanation of the overall metastasis action of the cells.

It has been further understood from analyzing the cell lines that the glycosylation process plays an important role in metastasis. In this particular research, the role of the distinct glycosylation within the E-selectin ligand CD44 has been probed. More information on glycosylation is detailed in the following section.

1.4 Functions of distinct glycosylations in E-selectin ligands

Glycosylation is the attachment of sugar molecules to proteins or lipids. Without glycosylation, some proteins do not fold as required to continue the biochemical pathways. Glycosylation also serves as a quality control epigenetic factor for glycoprotein folding within the endoplasmic reticulum. Apart from this, glycosylation plays an important role in the adhesion of cell molecules. Owing to these, a number of processes in the body are dependent on the glycosylation process. In many types of cancer, there are aberrances observed in specific genes, particularly glycosyltransferases, aiding in the glycosylation process. This leads to the cancer and tumor progression. These cause changes in the carbohydrate chain and further cause metastasis function in the body. This altered gene expression needs to be controlled in order to essentially stop the

aberrant glycosylation process. In other words, there is a need to control this aberrant glycosylation. Hence, the glycosylation pathway in the case of E-selectin ligands has been probed in this thesis to elucidate it in detail. Fundamentally, there are two kinds of these glycosylations. They are the N-linked glycosylation and the O-linked glycosylation[30]. In N-linked glycosylation, it has been found that the nitrogen atom binds to the oligosaccharide link. It has been found that oligosaccharyltransferase (OST) plays a prominent role in the binding of the oligosaccharide to the nitrogen atom[31]. N-linked carbohydrates structures are fundamentally N-linked carbohydrates that are made of N-acetyl glucosamine and amino acid asparagine[32]. The process of N-linked glycosylation is highly conserved and a unique process. In the case of O-linked carbohydrate, it has been found that they have covalent attachments to the proteins that provide a linkage between the structure of monosaccharide N-acetyl galactosamine and through the amino acids serine or threonine. In short, O-linked glycosylation is a sugar molecule that is attached to an oxygen atom in the amino acid residue within the protein. The structural differences pave the way for different biochemical processes between the two structures. Glycosylation during cancer formation even leads to the formation of tumor in the brain[33].

A number of empirical studies have been undertaken to showcase that there is a definitive role of the process of glycosylation in E-selectin ligands. Of this, the specific E-selectin ligand that has been discussed in this analysis is CD44[34]. Owing to their distinctive functionality, it has been found that they serve as effective cancer biomarkers. This serves in understanding the exact mechanisms involved carcinoma. CD44 function

is affected by glycosylation type. It has been found that each kind of the glycosylation has an impact on the CD44 mediated cell binding to hyaluronan (HA)[29].

CD24 is a small cell surface protein molecule anchored by glycosylphosphatidylinositol in many kinds of cancer[35]. It is has been found that they function as a signal transducer. It is otherwise known as the cluster of differencation 24 or heat stable antigen. CD24 (HSA)[36] is actually a protein that is encoded by the CD24 gene. Found on the chromosome 6, CD24 glycoprotein is found in the lymphocytes and some kinds of neuroblasts. Heavily glycosylated cell surface protein CD24 is important in the study of breast cancer. CD24+ cancer cells tend to spread more easily in bloodstream and seed new colonies in secondary organs[35, 37].

Every aspect of the glycosylation process can be changed or modified[38]. This includes the glycoside bond, glycerin composition, glycerin structure and also in the oligosaccharide layer. From this analysis, it is found that glycosylation plays an important biochemical process and is involved in a number of biochemical pathways in the body. The reason for exploring the function of glycosylation and the CD24 and CD44 gene expression is to understand the role of the glycosylation for the metastasis. Researchers have found that glycosylation plays an important role in the metastasis function. Details are discussed in the following.

1.5 Effects of N- and O-linked glycosylation inhibitors

Glycosylation inhibitors affect the glycosylation-dependent function of membrane proteins that will in turn affect adhesion between breast cancer cells and endothelial cells[39]. In other words, this has been found to be effective in essentially impeding the

metastasis function of the cells. Research has shown that the glycosylation inhibitors alter the N- and O-glycan patterns substantially, causing modification of the cells that lead to the impediment of carcinoma. There have been a number of investigations and analytical techniques that have been undertaken to find out about the definitive glycosylation inhibitors. It has been found that these glycosylation inhibitors in the body can be effective for the diagnosis and prognosis of cancer [40]. It has been well established that the therapeutic strategies can also be devised from formulating a mechanism for glycosylation inhibitors. One such line of research was undertaken for the regulation of apoptosis, revealing O-linked glycosylation is responsible for the ppGaNTases regulation of apoptosis[41]. These inhibitors function as effective epigenetic factors that lead to the control of the spread of cancer in the cells.

Deoxymannojirimycin (DMJ) has been found to be an effective inhibitor for prevention of cell adhesion between oligosaccharide layer of the viral and cellular N-linked glycoproteins[20]. It has been found that they can inhibit N-linked glycosylation by inhibiting mannosidase, which is a glycosylation processing enzyme[42]. When using DMJ on glycoproteins, glycoproteins' oligosaccharides are significantly altered, but DMJ doesn't change the surface expression of the glycoproteins themselves. In contrast, Benzyl-N-acetyl-α-galactosaminide (Bzl-GalNAc) inhibits elongation of O-glycans by inhibiting glycosyltransferase incorporation of glucosamine into O-glycans[43].

Hence, glycosylation inhibitors can serve an important function in preventing aberrant glycosylation. Due to this, a number of newer studies have been undertaken to ensure that this is controlled in the body. It has been found that DMJ, RAGE (the receptor

for advancedglycation endproducts) and mucin-type O-linked glycosylation are being investigated to solve the issue of aberrant glycosylation in the body.

1.6 Objective and specific aims

The objective of this investigation is to determine the type(s) of glycosylation(s) leading to E-selectin ligand function of cell surface proteins expressed by breast cancer cells.

Specific aim 1: Characterize E-selectin ligand activity on breast cancer cells. Cell adhesion molecule E-selectin is expressed by endothelial cells. E-selectin specifically binds to E-selectin ligands expressed by breast cancer cells. CD44 and CD24 are breast cancer stem cell markers. CD44, an E-selectin ligand plays an important role in breast cancer cell metastatic progression. Flow cytometry and western blot will be used. Various mAb, such as CD44 (515) and CD44 (2C5), will be used in flow cytometry to identify expression of E-selectin ligands on breast cancer cells. Using lysis buffer will lyse breast cancer cells. Membrane proteins will be tested using SDS-PAGE and western blotting to do immunoprecipitation studies.

Specific aim 2: To investigate glycosylation characteristics of E-selectin ligands on breast cancer cells and identify functions of N-linked glycosylation and O-linked glycosylation on E-selectin ligands. CD44 is a transmembrane glycoprotein that works as E-selectin ligands on some breast cancer cells. Extracellular interaction is involved in the attachment process of endothelial cells and breast cancer cells. Glycosylation changes of CD44 not only change conformation of CD44 but also functions of CD44. The influence of glycosylation to E-selectin ligand function will be measured by using

immunoprecipitation, western blot staining with various antibodies and flow cytometry. Cells will be treated with deoxymannojirimycin (DMJ) to prevent N-linked glycosylation of glycoproteins, whereas cells will be treated with benzyl-N-acetyl-α-galactosaminide (Bzl-GalNAc) to prevent O-linked glycosylation of glycoproteins[44]. Immunoprecipitation, western blot staining with various antibodies, flow cytometry, and real-time PCR will be used to determine the effect of N-linked glycosylation and O-linked glycosylation on E-selectin ligands.

Specific aim 3: To establish a mathematical model of breast cancer cells dynamic adhesion process to vascular endothelium. Under hydrodynamic forces of blood flow, breast cancer cell rolling is intermediated by the collaboration between E-selectin and E-selectin ligands on endothelial cells and breast cancer cells respectively. Breast cancer cells surface interaction with endothelium can be speculated by comparing to P-selectin interaction with P-selectin glycoprotein ligand-1, which is already well studied[25].

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell treatment

BT-20 cells were cultured with 2mM deoxymannojirimycin (DMJ) (Sigma-Aldrich, St. Louis, MO) for 48 hours to inhibit N-linked glycosylation of glycoproteins[44]. BT-20 cells were cultured with 1mM benzyl-N-acetyl-α-galactosaminide (Bzl-GalNAc) (Sigma-Aldrich) for 48 hours to inhibit O-linked glycosylation of glycoproteins[44]. BT-20 cells culture media is Eagle's Minimum Essential Medium (Invitrogen, Carlsbad, CA), with 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah).

2.2 Flow cytometry

Flow cytometry was used to determine surface glycoprotein expression on untreated BT-20 cells and BT-20 cells treated with DMJ or Bzl-GalNAc for 48 hours. Two-step labeling was used. Cells were incubated in 0.1% BSA/DPBS with Ca/Mg at a concentration of 10^7cells/mL. Antibody solutions were prepared in 0.1% BSA/DPBS with Ca/Mg at a concentration of 10μg/mL. Cells were incubated in primary antibodies for 30 minutes on ice, and after washing, cells were incubated in secondary antibodies for 30 minutes on ice then washed. Surface glycoprotein expression of the cells was tested using FACSAria Special Order Research Product flow cytometer (BD Biosciences, San Jose, CA).

2.3 Cell lysis and immunoprecipitation

Cells were lysated by using lysis buffer of 1% Triton X-100, 0.02% NaN_3, 150 mM NaCl, 0.5 mM Tris (pH 10.4), 1 mM EDTA and protease inhibitor cocktail (Roche

Applied Sciences, Indianapolis, IN)[45] at a concentration of 1million cells per 10μL lysis buffer. To immunoprecipitate E-selectin ligands in BT-20 cells, cell lysate was incubated with human IgG (h-IgG) isotype control (5 μg/10 million cell lysate) and protein G beads overnight at 4 ^{O}C on the rotator. Then the supernatant was incubated with E-Ig chimera (5 μg/10 million cell lysate) and protein G beads overnight at 4°C on the rotator. Protein G agarose was washed 4-5 times with DPBS with Ca/Mg. Elution buffer (5 mM EDTA, 50 mM Tris (pH 7.4), and 0.1% Triton-X-100) was incubated with agarose for 30 minutes at room temperature to release E-selectin ligands from protein G. Eluted samples were used to do SDS-PAGE and western blotting.

2.4 SDS-PAGE and western blotting

Cell lysates or immuoprecipitates were diluted with Laemmli reducing buffer and the separated on 4-15% Tris-HCl precast gels (Bio-Rad, Hercules, CA). Membrane proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad). FBS was used to block membranes overnight at 4°C. E-Ig chimera immunoprecipitations were stained with primary antibody anti-CD44 mAb (2C5) (R&D Systems, Minneapolis, MN) for one hour and then washed with Tris-buffered saline/0.1% Tween 20. The membrane was stained with appropriate secondary antibody[24]. β-actin was used as the western blot loading control.

2.5 Real-time PCR

Real-time PCR were performed on untreated, DMJ treated and Bzl-GalNAc treated BT-20 cell mRNA to determine the influence of enzyme treatment to gene of BT-20 cells. RNeasy plus kit (Qiagen, Valencia, CA) was used to extract and purify RNA.

Genomic DNA was removed by using DNaseI ((New England Biolabs, Ipswich, MA). High-capacity reverse transcription kit (Applied Biosystems) was used to reverse transcribe RNA from BT-20 cells. The cDNA was used to perform PCR. iCycler iQ5 real-time PCR instrument (Bio-Rad Laboratories) was used in this process. Forward and reverse primers (Integrated DNA Technologies, Coralville, IA) used to decide whether enzyme treatments change glycosylation gene expression levels are reported in Table 2[46].

Table 2. CD44 Primer sequences used in real-time PCR [47]

Primer	Sequence
CD44s forward	5' -CCT CCA GTG AAA GGA GCA GCA C-3'
CD44s reverse	5' -GTG TCT TGG TCT CTG GTA GCA GGG AT-3'
CD44v3 forward	5' -GTA CGT CTT CAA ATA CCA TCT CAG C-3'
CD44v3 reverse	5' -GGT GCT GGA GAT AAA ATC TTC ATC-3'
CD44v4 forward	5' -TTT CAA CCA CAC CAC GGG C-3'
CD44v4 reverse	5' -CAG TCA TCC TTG TGG TTG TCT G-3'
CD44v5 forward	5' -GTA GAC AGA AAT GGC ACC ACT GC-3'
CD44v5 reverse	5' -TTG TGC TTG TAG AAT GTG GGG TCT C-3'
CD44v6 forward	5' -TCC AGG CAA CTC CTA GTA GTA C-3'
CD44v6 reverse	5' -CAG CTG TCC CTG TTG TCG AAT GGG-3'
GAPDH forward	5'-AGC CAC ATC GCT CAG ACA C-3'
GAPDH reverse	5' -GCC CAA TAC GAC CAA ATC C-3'

2.6 Estimation of force on the tether bond

Adhesive force is the force between E-selectin on endothelial cells and E-selectin ligands on breast cancer cells, while disruptive force is formed by blood flow. The following equations are given by Goldman et al[48].

$$F \approx 1.7(6\pi rHS) \tag{1}$$

$$T \approx 0.94(4\pi r^3 S) \tag{2}$$

The force (F) and torque (T) exerted by blood flow during this process is related to local hydrodynamic environment, where r is radius of the cell and H is the distance from the cell center to the endothelial cell.

$$f\cos(180° - \theta) = F \tag{3}$$

$$f/\sin(180° - \theta) = T + rF \tag{4}$$

Knowing the hydrodynamic force F from equation 1, F is function of wall shear stress. F, angle θ between *f* and F, lever arm *l*, determines tether force *f*. The distance that a rapidly tethered cell moved in flow determines the lever arm *l*.

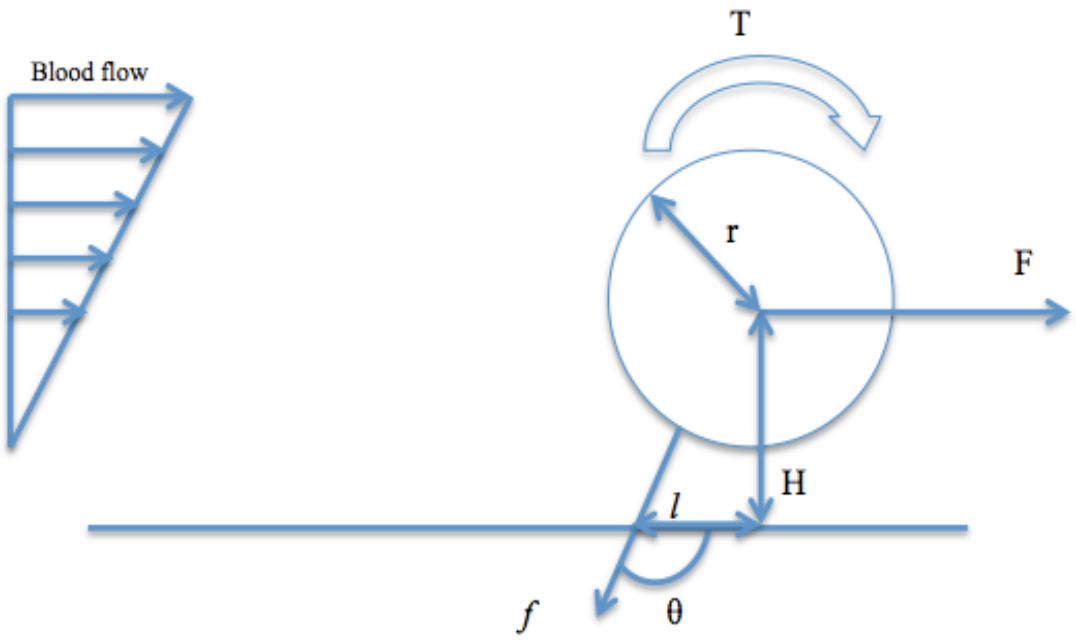

Figure 2. Schematic view of a cancer cell adhering in a shearing flow near blood vessel wall.

2.7 Calculation of cell hydrodynamic velocity

Before the establishment of a single bond between receptor and ligand, the slip velocity of the cell is constant and only depends on the shear rate and gap distance[49]. V is relative velocity between cell surface and blood vessel wall with units μm/s, $V = U - r\Omega$, where U is translational velocity with units μm/s and Ω is angular velocity with units radians per second. By equations given by Goldman et al[48]

$$\frac{U}{(r+h)S} \sim \frac{0.7431}{0.6376 - 0.200\ln(h/r)} \tag{5}$$

$$\frac{\Omega}{0.5S} \sim \frac{0.8436}{0.6376 - 0.200\ln(h/r)} \tag{6}$$

h is gap distance between cell and blood vessel wall, S is shear stress.

2.8 Estimation of shear-induced on-rate

Convection is the dominant factor for the binding rate of E-selectin and E-selectin ligands. Shear-induced on-rate for selectin-ligand bonds must be high enough so that a rapidly moving cancer cell can form bonds with endothelial cells. E-selectin can form catch bonds with its ligand. Compressive load can certainly increase the intrinsic reaction rate of selectin-ligand bond formation. Applied force can influence shear-induced on-rate. Using shear-induced on-rate equation given by [25, 49, 50]

$$k_+ = 2DP_e\left(\frac{8a^2k_{in}}{3\pi DP_e + 8a^2k_{in}}\right) \quad (7)$$

k_{in} is the intrinsic association rate of a ligand-receptor pair within the reactive circle. Péclet number is defined $P_e = a \cdot \mathbf{v}/\mathrm{D}$. Subbing in the expression for relative velocity gives Pe=a · (U-rΩ)/D, where typical values for the lateral diffusion $D=10^{10}cm^2/s$ and typical radius of the receptor radius $a=10^{-7}$ cm[49].

CHAPTER 3: RESULTS

A portion of this work has been published previously[47].

3.1 BT-20 cells have CD44+/CD24+ expression

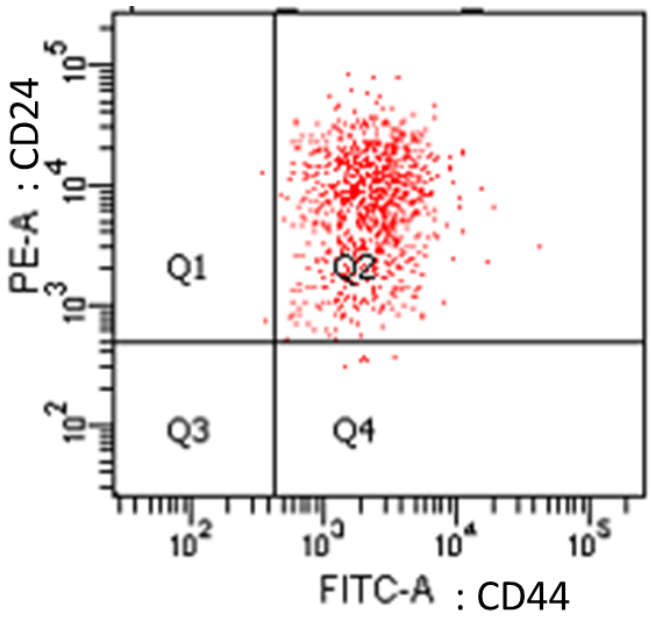

Figure 3. Expression of CD44 and CD24 on BT-20 cells.

BT-20 cells simultaneously labeled with CD44 and CD24 mAb were analyzed by flow cytometry to screen for expression of cell surface molecules CD44 and CD24. Cell lines that have CD44+/CD24- expression are stem cell-like. Otherwise, cell lines are non-stem cell-like (CD44+/CD24+, CD44-/CD24+, CD44-/CD24-)[51]. BT-20 cells are from human invasive ductal carcinoma of a 74-years old Caucasian female patient. BT-20 cells can express CD44 that have E-selectin ligand activity while CD44 expressed by another breast cancer cell line, MDA-MB-231, don't have E-selectin ligand activity[47].

3.2 DMJ and Bzl-GalNAc treatment flow cytometry results

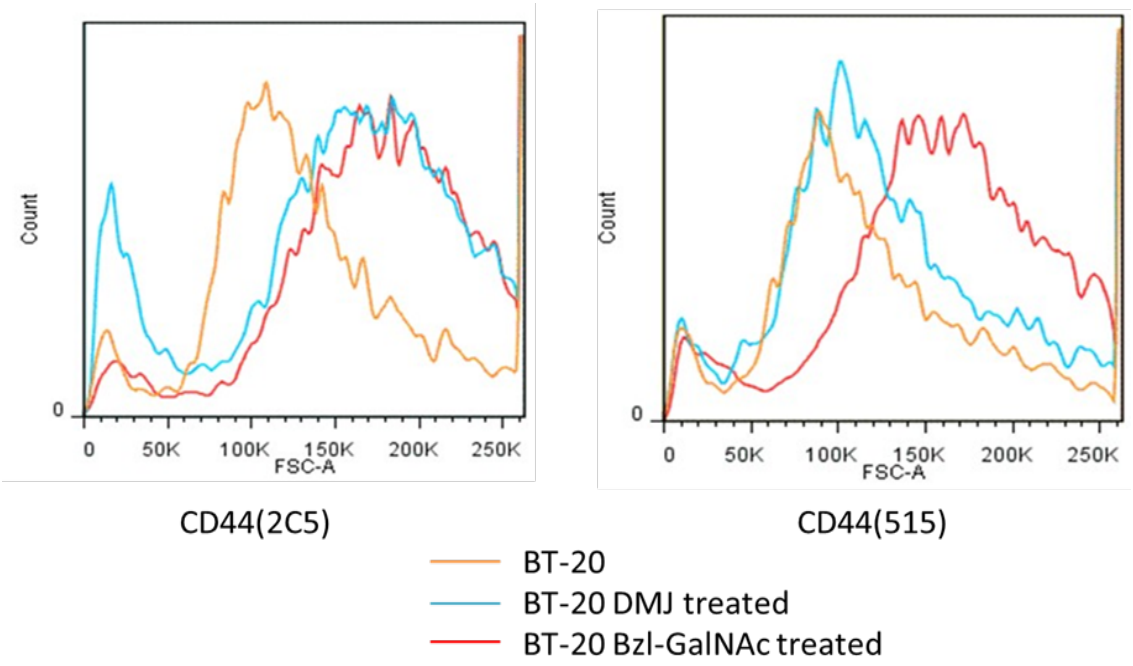

Figure 4. Flow cytometry analysis of CD44 (2C5) and CD44 (515) surface expression on BT-20 cells and BT-20 cells treated with 2mM concentration DMJ or 1mM concentration Bzl-GalNAc for 48 hours. Data are representative of N=2 independent experiments.

After BT-20 cells DMJ and Bzl-GalNAc treatments, untreated BT-20 cells and BT-20 treated cells were all tested using flow cytometry. DMJ and Bzl-GalNAc treatment increase both CD44 (2C5) and CD44 (515) expression of BT-20 cells.

3.3 Glycosylation inhibitors treatment effects on CD44 expression of BT-20 cells

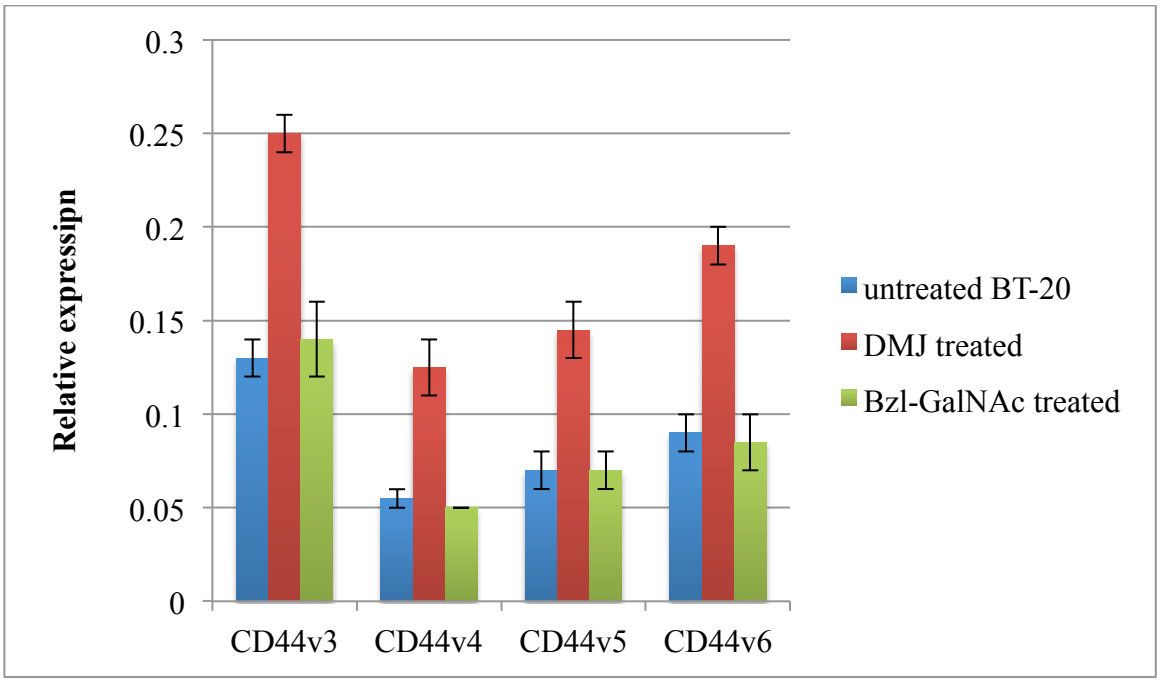

Figure 5. Real-time PCR is performed on untreated, DMJ treated and Bzl-GalNAc treated BT-20 cell mRNA to determine the influence of enzyme treatment to gene of BT-20 cells. Expression is relative to the housekeeping gene GAPDH. Error bar denote standard error, N=2.

Comparing the differences by using One-way ANOVA (ANalysis Of VAriance) with post-hoc Tukey HSD (Honestly Significant Difference) Test Calculator, no statistically significant difference was found among the means of treated or untreated BT-20 cells. BT-20cells express multiple CD44v isoforms and they are E-selectin ligands[52]. The result shows glycoslation inhibitors treatments do not have significant effects on CD44 expression of BT-20 cells.

3.4 DMJ and Bzl-GalNAc treatment western blot results

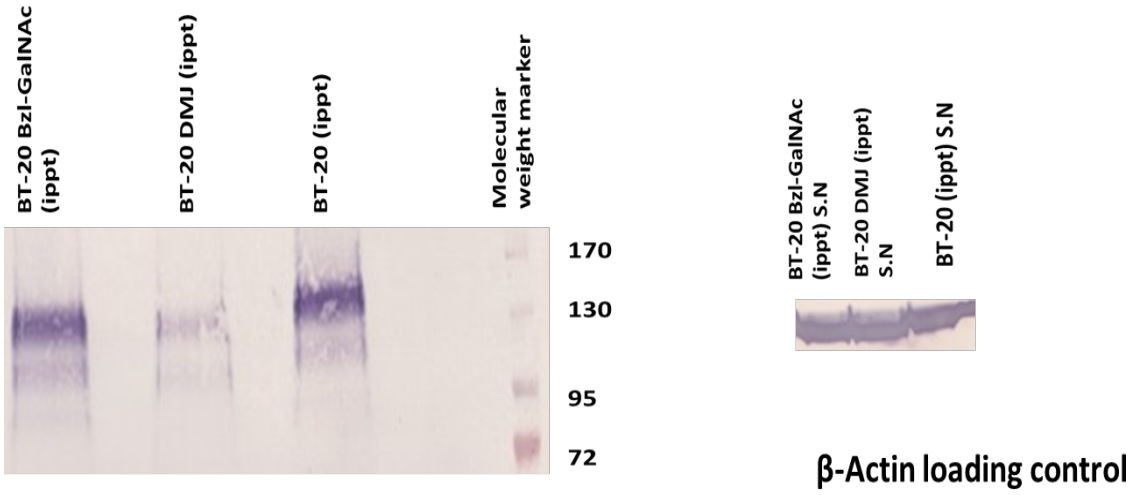

Figure 6. E-Ig chimera immunoprecipitations from 10^7cells were western blotted with anti-CD44 (2C5) mAb. β-actin loading controls are supernatant from immunoprecipitate (ippts). Data are representative of N=2 independent experiments.

In previous research[52], there were three distinct CD44 bands at ~95, ∼110, and ∼150 kDa appearing in BT-20 lysates. CD44s is presumed to be the band at ∼95 kDa, and bands at >100 kDa are most likely CD44v[52]. So in the western blotting result of E-Ig immunoprecipitation shown in s, the bands are CD44v. DMJ treated BT-20 cells have a much lighter band in western blotting outcome, because they have less bonding to E-selectin. The bonding to E-selectin does not change much for Bzl-GalNAc treated BT-20 cells, since the intensity of the band does not change much from untreated BT-20 band.

DMJ treated BT-20 cells have significant decrease of CD44v E-selectin ligand activity while Bzl-GalNAc treated BT-20 cells don't have much affect on CD44v E-selectin ligand activity. This leads to the result that N-linked glycosylation is important for CD44v E-selectin ligand function.

3.5 Mathematic model for BT-20 cells dynamic adhesion process to endothelium

For BT-20 cells, the radius is about 10 μm[53]. To calculate the tether force between a BT-20 cell and endothelial cells, the lever arm l is needed to determine angle θ between tether force f and disruptive force F. Due to the lack of studies about BT-20 cells hydrodynamic behavior, we lack data to know the lever arm l of BT-20 cells. Thus, with the present data, we are unable to calculate the force on the tether bond. However using literature values, a shear-induced on-rate k_+ value can be estimated.

For the gap distance between BT-20 cell and blood vessel wall, it is assumed to be an unstressed leukocyte microvillus's length, h=3μm[25]. For shear rate ranging from $50s^{-1}$ to $80s^{-1}$ based on real blood vessel hydrodynamic conditions, Péclet number is about 12.83-20.53 for BT-20 cells.

Table 3. Parameters used in mathematic model

Parameter	BT-20
r, μm	10[53]
h, μm	0.3[25]
$x_{\beta,}$ Å	1[54]
D, $\mu m^2/s$	0.01[49]
a, μm	0.001[49]
k_{in}, s^{-1}	1000[49]

$k_+ = 2\times10^{-5}Pe\left(\frac{1}{11.775Pe+1}\right)\mu m^2/s$, for BT-20 cells, Pe=0.25665s×S, S is shear rate with units s^{-1}, $k_+ = 5.133\times10^{-6}\mathrm{S}\left(\frac{1}{3.02\mathrm{S}+1}\right)\mu m^2$.

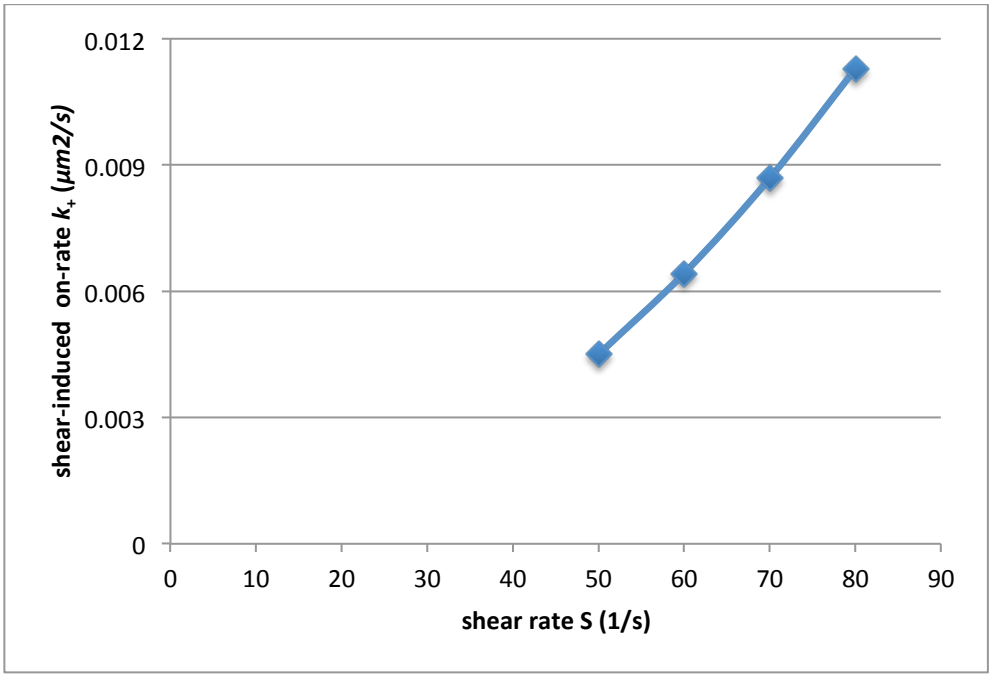

Figure 7. Estimation of shear-induced on-rate k_+ as a function of shear rate ranging from $50s^{-1}$ to $80s^{-1}$ for BT-20 cells.

For BT-20 cells, shear-induced on-rate k_+ increases with increasing shear rate during shear rate from $50s^{-1}$ to $80s^{-1}$.

CHAPTER 4: DISCUSSION AND CONCLUSIONS

Flow cytometry shows that DMJ and Bzl-GalNAc treatment increase both CD44 (2C5) and CD44 (515) expression of BT-20 cells. Western blot results reveal that DMJ treatment decreases E-selectin ligand activity of CD44 of BT-20 cells. N-linked glycosylation of glycoproteins may have a significant effect on E-selectin ligand function of cell surface proteins, particularly CD44 variants, expressed by breast cancer cells.

The reason for studying breast cancer and metastasis is that understanding the level of progression of cancer leads to developing better survival rates and prolonging life expectancies.

BT-20 cells express CD24. It has been found that CD24 is often associated with the prognosis of various cancers. The exact heuristics and the mechanism adopted by CD24 are not clear. From an analysis of breast cancer tissues, it was found that the CD24 was used in the DNA methylation and histone modification status in the promoter region of CD24. It was also found that the patients who had higher levels of CD24 had lower survival rates than patients who had low CD24[55] and from analysis that the gene expression of CD24 was involved in histone acetylation. This is an independent processing of the DNA methylation[56]. This points to the mechanism of action of the epigenetic regulation of CD24. The result indicated that over gene expression of the CD24 gene causes lower survival rates[57]. Thus analysis of CD24 can be a potentially strong therapeutic target for the specific subtypes of breast cancer. CD24 is thus found to be more adept in diagnosing carcinoma owing to their enhanced functionality. CD24 on

BT-20 cells has not yet been evaluated as an E-selectin ligand, so future work is needed to obtain better understanding of CD24.

From shear-induced on-rate, we can obtain the time that is needed for a bond to form. Shear-induced on-rate increases with an increasing compressive force, from which we can know that load influences on-rate as well as off-rate[27]. The binding rate is first order with respect to the local ligand site density on the substrate, and the intrinsic association rate between receptors and ligands is independent of hydrodynamic conditions.

It's important that the bond off rate is high enough to cause the cancer cell rolling. Different adhesive molecular bonds have different kinetic properties[27].

$$k_{off} = k_{off}^{0} exp\left(\frac{x_\beta f}{k_B T}\right) \tag{8}$$

k^0_{off} is bond off-rate without any affected force, which is unstressed k_{off}, k_{B} is the Boltzmann's constant, T is the absolute temperature, f is tether force, and x_β is a parameter with units of length. The most used method to measure x_β is atomic force microscopy (AFM), by coating an AFM tip with ligand molecules and counter surface with matching receptors. The AFM tip is allowed to approach the counter surface where the molecules can bind, and then the tip is consequently withdrawn at a constant pulling velocity[54]. The movement of the cantilever is monitored during the process so that the forces between receptors and ligands can be studied.

For catch–slip bonds[27],

$$k_{off} = k_c^0 \exp\left(\frac{x_c f}{k_B T}\right) + k_s^0 \exp\left(\frac{x_s f}{k_B T}\right) \tag{9}$$

For catch-slip pathways, k_c^0 is the unstressed bond dissociation. x_c and x_s are parameters with units of length for catch and slip pathways respectively.

For E-selectin and E-selectin ligands binding kinetics,

$$N_u + N_l \underset{k_{off}}{\overset{k_+}{\rightleftarrows}} N_b \tag{10}$$

N_u is unbound number of cells, N_l is number of ligands at contact area, which can be calculated from ligands density m_l at contact area and lever arm length l. N_b is the bound cells number. Total number of cells $N_T = N_u + N_b$, $\frac{N_b}{N_T} = \frac{K_+ N_l}{k_{off} + k_+ N_l}$.

$$\frac{N_b}{N_T} = \frac{1}{\frac{k_{off}^0 \exp\left(\frac{x_\beta f}{k_B T}\right)}{k_+ N_l} + 1} \tag{11}$$

For the same type of selectin-ligand bond at time t, k_{off}^0, N_l, $k_B T$, x_β, which depend on bond type, are constant, and tether force f is a function of shear stress S at time t. So binding ratio N_b/N_T is a function of shear rate S for a given bond type.

From previous data[53] shown in Figure 8, BT-20 cells binding to EpCAM under flow condition, we can obtain that binding ratio is associated with shear rate, such that binding rate decreases with increasing shear rate. The adhesive force between BT-20 cells and EpCAM is smaller than that between BT-20 cells to E-selectin[47]. But it is still believed both processes follow similar hydrodynamic behavior. From the study about BT-20 cells binding to EpCAM, we can assume binding ratio for CD44 in BT-20 cells to E-selectin on endothelium also decreases with increasing shear rate. k_{off}^0, lever arm length l and ligand density m_l at contact area need to be determined by future experiments to calculate binding ratio for BT-20 cells to E-selectin.

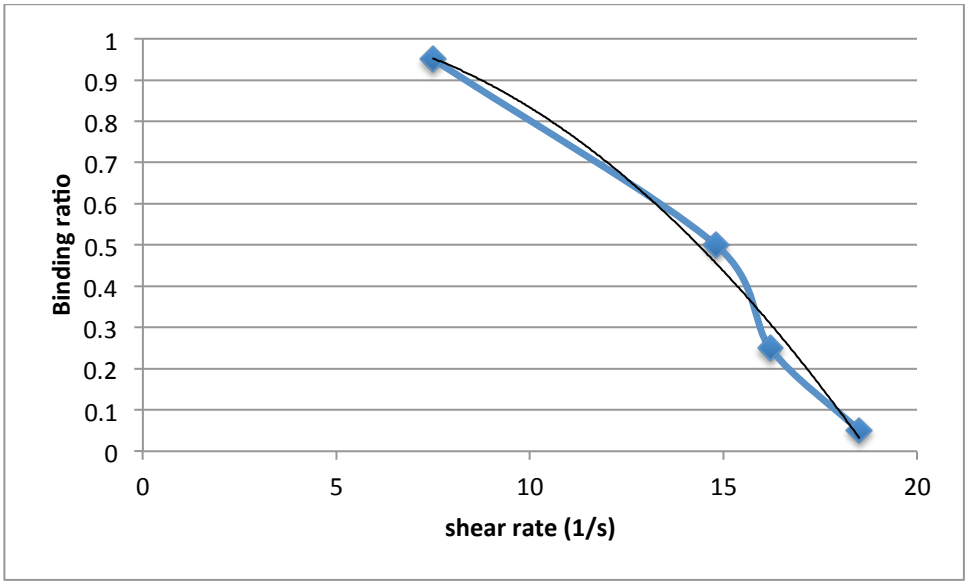

Figure 8. BT-20 attachment ratios to EpCAM under flow conditions[53].

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