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The Role of Breast Cancer Associated MUC1 in Tumor Cell Recruitment to Vascular Endothelium During Physiological Fluid Flow

by

Garnet James Horne

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Experimental Pathology

Department of Laboratory Medicine and Pathology

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *The Role of Breast Cancer Associated MUC1 in Tumor Cell Recruitment to Vascular Endothelium During Physiological Fluid Flow*, submitted by Garnet James Horne in partial fulfillment of the requirements for the degree of Master of Science in Experimental Pathology.

Dr. Judith Hugh (Supervisor)

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Date Approved: June 14/99

Dedication:

This is dedicated to the hard work and efforts of my mother and father. Thank-you both.

Abstract:

Mortality in cancer is due to metastasis, the ability of cancer cells to gain access to the circulation then exit and proliferate at a secondary site in the body. This process is mediated by complementary adhesion molecules on the circulating cell and vasculature. The breast cancer mucin, MUC1, is a glycosylated transmembrane protein that binds the vascular ligand Intercellular Adhesion Molecule 1 (ICAM-1) in static conditions. This study reports that MUC1-expressing tumor cells bind ICAM-1 positive endothelial monolayers under dynamic physiological fluid flow conditions. Antibodies to both ICAM-1 and MUC1 inhibit this adhesive process. However, the binding appears to be complex with a contextual requirement for other proteins since adhesion could not be inhibited by competition with soluble or liposomal MUC1. Immobilized recombinant human ICAM-1-immunoglobulin fusion protein (rhICAM-1) could not support adhesion under similar conditions. This suggests that MUC1/ICAM-1 binding under shear stress may involve an adhesion cascade, similar to inflammatory cells.

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List of Abbreviations

1. AM	Acetoxymethyl ester
2. BCECF	$2^1,7^1$-bis-(carboxyethyl)-5-(and 6)-carboxy fluorescein
3. BSA	Bovine serum albumin
4. cAMP	$3^1,5^1$ cyclic adenosine monophosphate
5. CDS	Cell dissociation solution
6. DAB	$3,3^1$-damino-benzidine
7. DH_2O	Distilled water
8. DPBS	Dulbecco's phosphate buffered saline
9. E-cadherin	Epithelial cadherin
10. E-selectin	Endothelial selectin
11. ECGS	Endothelial cell growth supplement
12. EGF	Epidermal growth factor
13. ELISA	Enzyme linked immunosorbent assay
14. FVIII	Factor VIII
15. FACScan	Fluorescent activated cell sorting scan
16. FCS	Fetal calf serum
17. fMLP	fMet-Leu-Phe
18. GAH	Goat anti-human IgG Fc specific
19. GAM	Goat anti-mouse
20. HBSS	Hank's balanced salt solution
21. HRP	Horse radish peroxidase
22. HUVECs	Human umbilical vein endothelial cells
23. ICAM-1	Intercellular adhesion molecule - 1
24. Ig	Immunoglobulin
25. IL-1β	Interleukin-1 Beta
26. IL-4	Interleukin-4
27. IS	Immunoglobulin Superfamily

28. L-selectin	Lymphocyte selectin
29. LCA	Leukocyte common antigen
30. LFA-1	Lymphocyte function-associated antigen
31. Lu-ECAM-1	Lung derived endothelial cell adhesion molecule
32. M199	Medium 199
33. mAbs	monoclonal antibodies
34. Mac-1	CD11b/CD18
35. MFG	Milk fat globule
36. MHC	Major histocompatibility antigen
37. NBT/BCIP	Nitroblue tetrazolium chloride/5-bromo-4-chloro-5-indoyl phosphate
38. P-selectin	Platelet selectin
39. PBS	Phosphate buffered saline
40. PE	Phycoerythrin
41. PECAM	Platelet-endothelial cell adhesion molecule
42. PKC	Protein kinase C
43. PSGL-1	P-selectin glycoprotein ligand - 1
44. rhICAM-1	recombinant human ICAM-1-IgG fusion protein
45. SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
46. $sLe^{x/a}$	sialyl Lewis X and A antigen
47. sMUC1	soluble MUC1
48. TBS	Tris buffered saline
49. TMB	$3,3^{1},5,5^{1}$-tetramethyl benzidine dihydrochloride
50. TNF-α	Tumor necrosis factor Alpha
51. VCAM-1	Vascular cell adhesion molecule - 1
52. VLA-4	Very late antigen - 4

Chapter 1

INTRODUCTION

1.1 Background

Cancer is the uncontrolled clonal proliferation of a genetically mutated cell which has gained the ability to spread to distant sites in the body from a primary site of growth. It is a major cause of death and is the leading cause of lost potential years of life in Canada.[1] Breast cancer, which affects one in ten North American females[1], causes death in approximately 25% of those afflicted.[2] Mortality in breast cancer derives from the ability of tumor cells to metastasize or disseminate to distant vital organs from their primary environment of growth and thereby evade the surgical and radiotherapeutic treatments aimed at the primary site. Insight into the mechanisms of tumor cell metastases may lead to therapies aimed at preventing malignant cells from disseminating which would allow for more successful treatments and possible cures for cancer patients of the future.

1.2 The Metastatic Cascade

Metastasis is a multi-step cascade of events whereby tumor cells migrate from the initial site of growth to various secondary locations throughout the body (Figure 1).[3] Tumor cells must first invade from their *in situ* environment through the basement membrane into the underlying interstitial stroma of the tissue. This occurs either through the release of matrix metalloproteinases and collagenases or by stimulating the stromal cells to modulate their secretions.[4] These invasive epithelial cells may then migrate into the surrounding capillaries and lymphatics of the primary tissue. This process, known as intravasation, is facilitated by tumor induced neo-angiogenesis. As a tumor grows an increased oxygen demand is met by the formation of new, poorly constructed capillaries

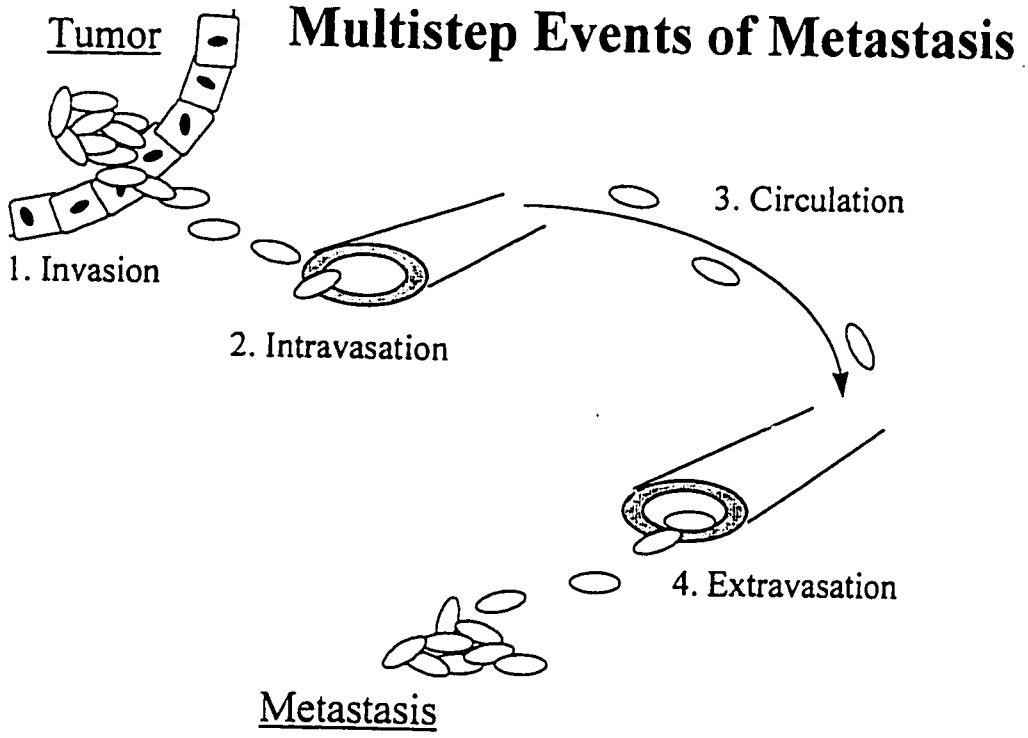


Figure 1. The metastatic cascade consists of numerous sequential events. For a primary tumor to successfully metastasize and proliferate at a distant site each step of the cascade must be complete. The steps include invasion and migration, intravasation, circulation and immune evasion, extravasation, and secondary proliferation.

which are often the site of tumor cell entry into the circulation.[5] Once in the blood stream cells come to full arrest in a distant organ capillary bed. After arrest it is believed that tumor cells extravasate through the vascular endothelium and basement membrane using similar degradative mechanisms described during the initial invasion. The tumor cells then invade into the underlying stroma of the secondary tissue and again begin to proliferate uncontrollably, eventually destroying the secondary organ, causing complications or death of the patient. Each stage of the metastatic cascade provides potential targets that may be exploited to prevent tumor dissemination.

1.3 Extravasation From Circulation

1.3.1 What is Extravasation?

Extravasation refers to a highly complex and coordinated process by which a circulating cell leaves the vasculature and migrates into the underlying stroma. This process occurs frequently as part of normal immunity as inflammatory cells selectively extravasate in order to prevent or respond to infection in any tissue. Studies of immune cell extravasation have led to a general model of active migration out of the circulation via adhesion molecules (Figure 2). Since adhesion molecules involved in inflammatory cell extravasation are often expressed on tumor cells,[6,7,8] this provides an excellent model from which to study the similar process of tumor cell extravasation.

1.3.2 The Inflammatory Cell Adhesion Cascade

The immune system regulates inflammatory cell migration towards infected tissues where infectious agents are selectively destroyed without harm to the host tissue.

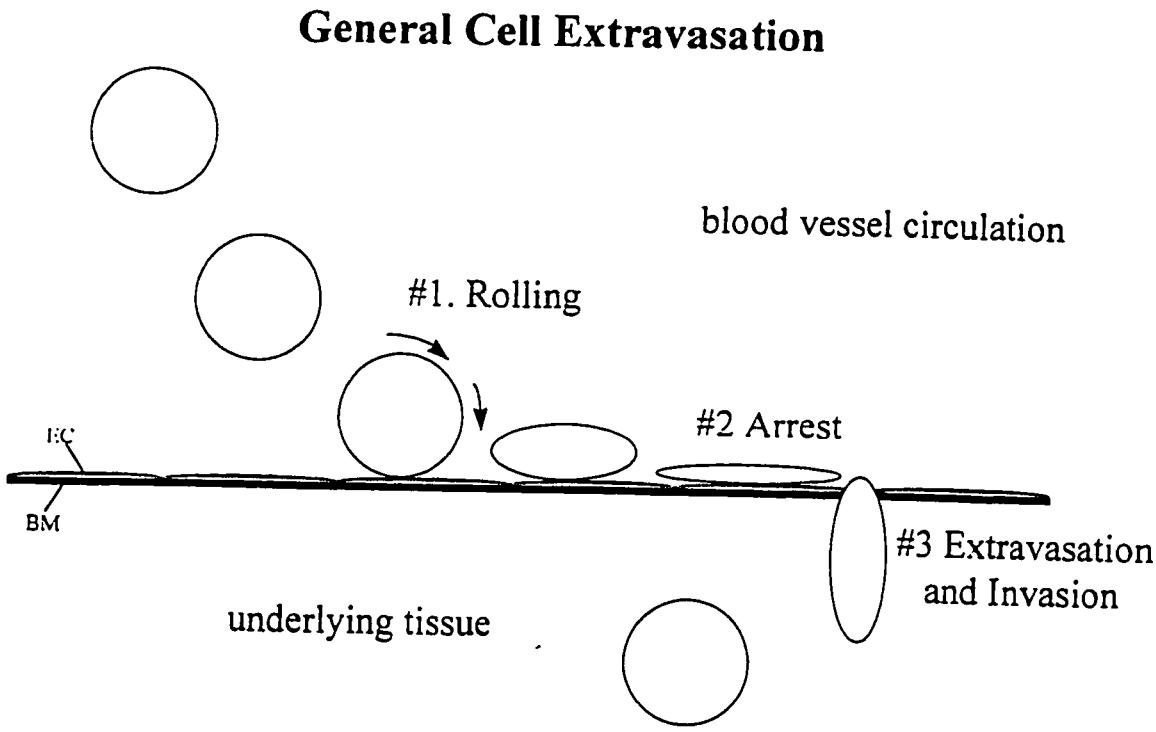


Figure 2. A general cascade model for how a circulating cell actively extravasates. The cell in circulation will begin to roll or "tether" via transient adherence with the endothelium (step #1). Rolling is followed by high affinity adhesion causing arrest of the cell on endothelium (step #2). Firm adhesion then triggers migration through the endothelium and basement membrane (step #3).

To accomplish this goal a "Lock and Key" mechanism exists between inflammatory cells and the capillary endothelium which vascularizes all tissue.

When a foreign agent is introduced into a tissue, immune mediators such as cytokines, endotoxins and chemoattractants are released from the damaged tissue.[9] This leads to the upregulation of specific adhesion molecules on the endothelium of perfusing capillaries.[9-11] Blood borne inflammatory cells, such as lymphocytes and granulocytes, which possess the appropriate ligands for the upregulated endothelial molecules are recruited to the surface of the endothelium through an adhesion cascade consisting of three basic sequential steps (Figure 3).[9,10,12] Inflammatory cells first begin to transiently roll or "tether" to endothelial cells which reduces the circulating cells' velocity exposing it to cytokines diffusing from the infected tissue.[12-14] This exposure activates the leukocyte's secondary adhesion molecules, allowing for the second stage of high affinity binding and cell arrest on the endothelium. Finally, immune cells must invade the underlying tissue by migrating through the endothelial cell layer and basement membrane. After full extravasation the immune effector cell can then phagocytize and destroy the original infecting particle.

1.3.3 Adhesion Molecules of Inflammatory Cell Extravasation

There are three main families of adhesion molecules that participate in normal immune cell recruitment.[9,10] These are the Selectins, Integrins, and members of the Immunoglobulin Superfamily (IS).[9,10,12,13] A brief review of each family's structure, function and regulation will be presented in the context of their participation in the extravasation process.

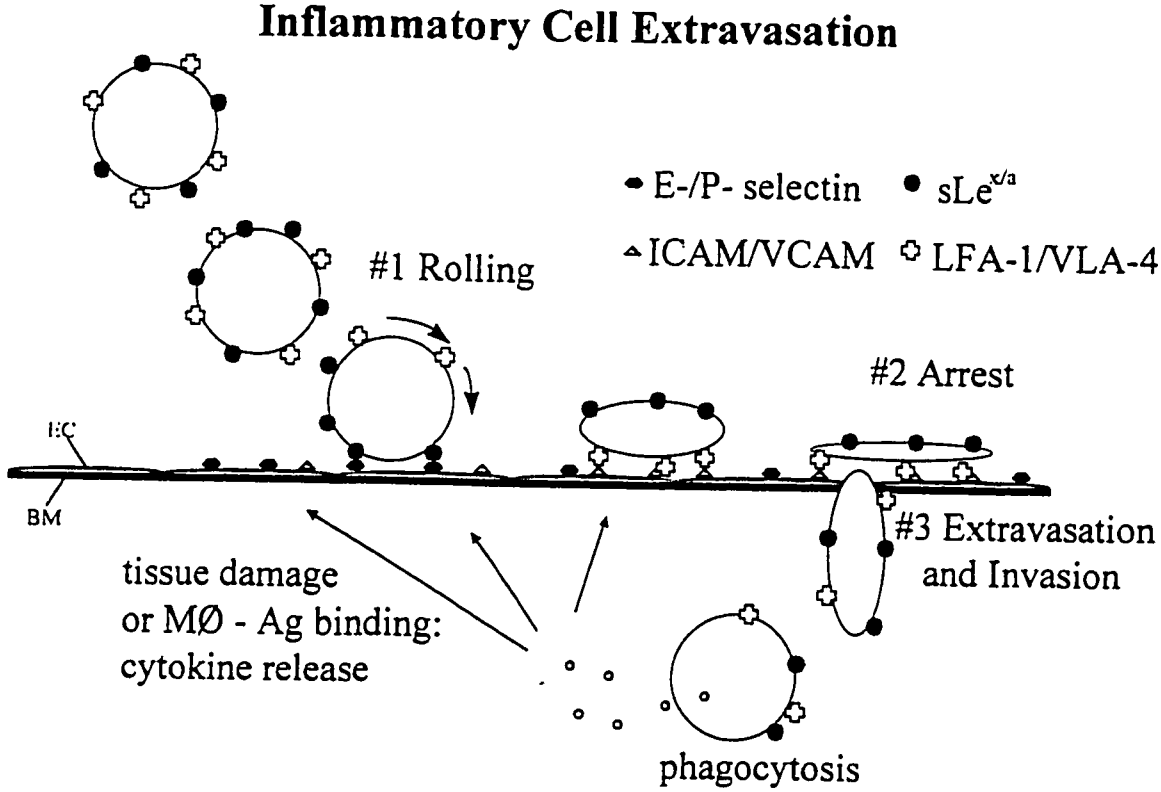


Figure 3. Inflammatory cell extravasation uses specific adhesion molecules to complete the extravasation cascade. An infecting agent upregulates endothelial adhesion molecule expression by release of cytokines and endotoxins. Immune cell carbohydrate ligands mediate rolling by adhesion to endothelial selectins (step #1). Activation of the integrins (LFA-1/VLA-4) then mediates high affinity binding to endothelial IS members (ICAM's/VCAM's) (step #2). The integrin/IS adhesion mediates extravasation into the tissue where the infecting particle is phagocytized and destroyed by the immune cell (step #3).

A) Step 1: Tethering

i) The Selectins

Transient "tethering" or rolling, which is the first phase of inflammatory cell recruitment is mediated via the selectins and their carbohydrate ligands (Figure 3). The selectins are divided into E-selectin (endothelium), P-selectin (platelet), and L-selectin (lymphocyte) according to the site of original identification.[10,15] Each protein consists of a carbohydrate-binding NH_2 terminal lectin-like domain, an epidermal growth factor (EGF) repeat and a unique number of modules containing approximately 66 amino acids each (Figure 4).[15] The lectin domain of each selectin is greater then 60% identical and has a Ca^{2+}-dependent binding function.[15] The carbohydrate ligand categories include (a) oligosaccharides related to sialyl Lewis x (sLe^x) and sialyl Lewis a (sLe^a); (b) phosphorylated mono- and polysaccharides; and (c) sulfated polysaccharides.[10] All three selectins bind the oligosaccharides ($sLe^{x/a}$) which have been the best characterized ligands due to their similarities in structure and function.[9,10] Though all three types are found on various circulating cells, only E- and P-selectin are expressed on the endothelial surface at sites of inflammation.[14]

The E-selectin family member has been the most widely studied. It is expressed above basal levels on endothelium after 4 hours of cytokine stimulation but steadily decreases in expression beyond this time point.[9,13,16] At maximal levels, E-selectin mediates rolling and possibly migration of neutrophils,[17] monocytes, T-lymphocytes, eosinophils and basophils.[18-21]

P-selectin is stored within endothelial Weibel Palade bodies[14] and is rapidly redistributed to the cell surface upon stimulation with soluble immune mediators.[15] P-

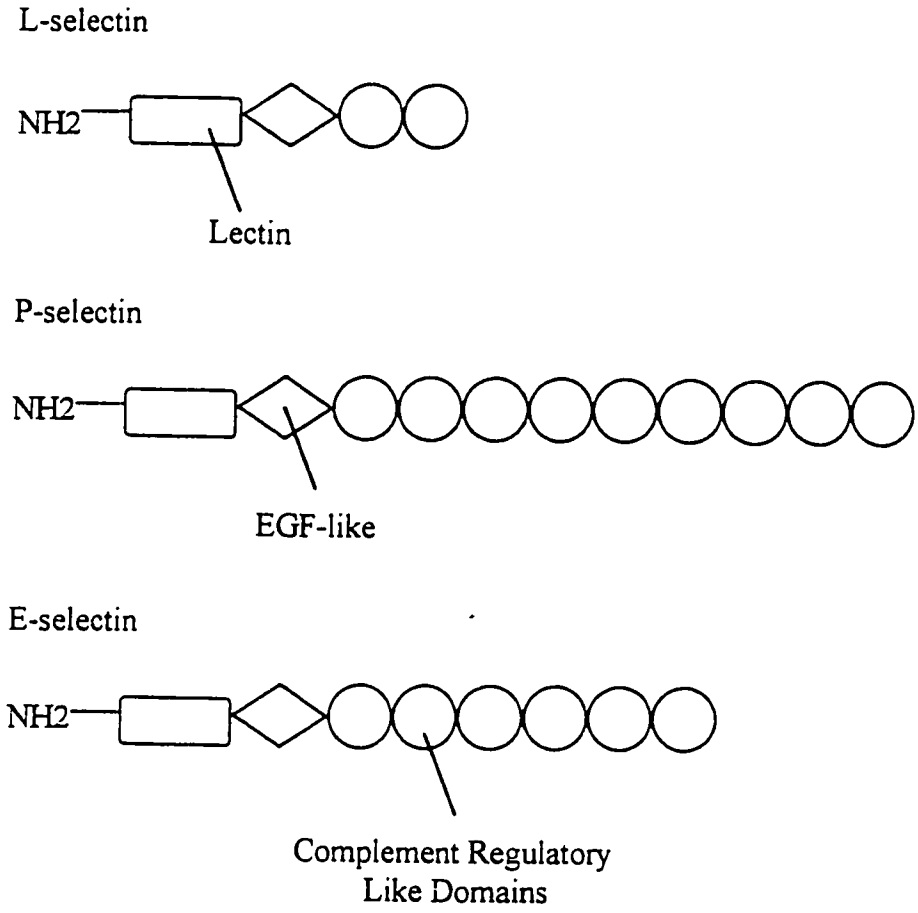


Figure 4. Selectin molecules are similar in structure and function varying only in the number of 66 amino acid modules in each. Each has a known function in adhesive processes with common carbohydrate ligands.

selectin ligands are often similar to those of E-selectin, and it is therefore capable of supporting rolling and recruitment of most leukocytes and lymphocytes to sites of inflammation via transient rolling.[15] Deficiencies of only one selectin result in a normal phenotype demonstrating the redundancy of function between these molecules.[13] In mice deficient in both E- and P-selectin, severe leukocytosis and bacterial susceptibility is evident.[13] The selectin carbohydrate ligands are also equally important to immune cell targeting. Human patients with impaired fucosylated carbohydrate synthesis (Leukocyte Adhesion Deficiency type II) necessary for producing selectin ligands demonstrate impaired leukocyte rolling on endothelium and clinically have an increased susceptibility to infection.[13]

B) Step 2: Firm Adhesion and Arrest

The second stage of inflammatory cell extravasation occurs through high affinity binding of integrins on blood borne cells to IS ligands on the endothelium (Figure 3). This high affinity interaction leads to the arrest of the cells subsequent to their rolling on the stimulated endothelial cells.[22,23]

i) The Integrins

All integrins have a common Alpha Beta ($\alpha\beta$) heterodimer structure which spans the plasma membrane.[24] Integrins are classified into families according to which of the 8 known β subunits are present in the dimer. There are 15 known α subunits and although there are many potential $\alpha\beta$ combinations, α chains will preferentially associate with only specific β chains, such that only 20 $\alpha\beta$ integrin combinations have been described.[10,24] The $\alpha\beta$ association is mediated by the extracellular domain of each

subunit.[24] The extracellular domain of all the α subunits have approximately 7 tandem repeats of a 60 amino acid sequence which interact with divalent cations vital to its function.[10] The site of ligand binding is most often present in a 180 amino acid sequence flanking the tandem repeats.[10] The β chain also has a tandem repeat region, rich in cysteine, that is important for maintaining the tertiary structure of the heterodimer.[10] The cytoplasmic domain of each α and β subunit generally consists of less than 50 amino acids.[24] Although cytoskeletal and cytoplasmic protein associations have been demonstrated with most β subunits, only certain α chains harbor similar associations due to a high degree of variability in the α chain cytoplasmic sequence.[22] These associations suggest that a major function of integrins is to mediate cytoskeletal and cytoplasmic alterations upon extracellular adhesion.[24,25]

There are 2 major leukocyte integrin families (β_1 and β_2) that mediate adhesion to endothelium. The β_2 (CD18) subunit associates with 1 of 3 possible α chains (CD11 a, b, c),[9] the best characterized being CD11b / CD18 (Mac-1) and CD11a / CD18 (LFA-1).[9] Mac-1 and LFA-1, which are expressed on activated lymphocytes, neutrophils, monocytes, and natural killer cells bind similar protein ligands, such as intercellular adhesion molecules (ICAM's) of the IS, and mediate adhesion and migration.[21] Patients with deficient β_2 expression (Leukocyte Adhesion Deficiency type I) show increased susceptibility to infection.[13] Ligation of the β_2 integrins is associated with cell motility and changes in the intracellular Ca^{2+} levels.[24,26] The β_1 integrin family member (VLA-4) found on lymphocytes, eosinophils, basophils, natural killers and monocytes binds the IS molecule, vascular cell adhesion molecule-1 (VCAM-1).[9,10,24] This interaction mediates T

cell co-stimulation, regulation of cytoplasmic pH, and endothelial cell/immune adhesion.[24]

Both leukocyte integrin families are subject to similar mechanisms of regulation. They are normally expressed at a constant basal level. Integrin-mediated binding is regulated through conformational changes in the molecule (affinity modulation) rather than an increase in the number of molecules expressed (frequency modulation).[9, 27-29] Inflammatory mediators such as tumor necrosis factor (TNF), complement factor 5a and fMet-Leu-Phe (fMLP) trigger the conformational change. Binding affinities are also partially mediated by divalent cations and the presence of lipid which contribute to the molecular conformation.[24] Intracellular mechanisms including the involvement of protein kinase C (PKC) also play a role in affinity modulation via phosphorylation of specific cytoplasmic sequences on the molecule.[24]

ii) The Immunoglobulin Superfamily

The IS are the endothelial ligand component necessary for firm immune cell adhesion to endothelium. These glycoproteins are characterized by the presence of a di-sulfide-bridged loop immunoglobulin domain which mediates adhesive interactions (Figure 5).[9,10,30] The number of Ig-like domains varies between members of the IS family.[9] The VCAM and ICAM subfamilies are implicated in recruitment of circulating cells to endothelium.[9,10] Though each subfamily binds distinct ligands, each molecule's structure, function and regulation are similar and will be discussed in terms of the IS member, ICAM-1.[9,10]

The ICAM-1 molecule is a 100kD protein possessing five glycosylated Ig-like domains (D 1-5).[10] Each Ig-like domain is encoded by a separate exon found on

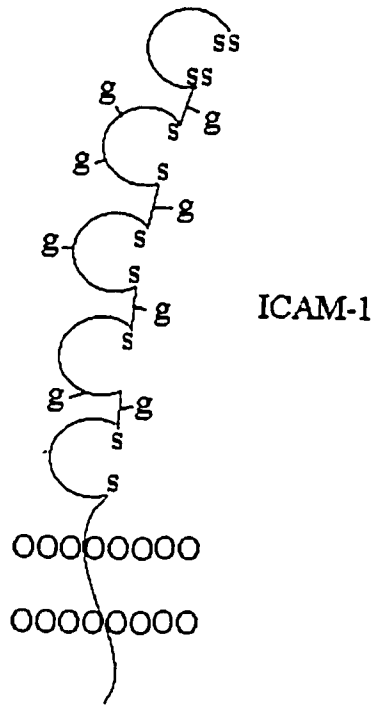


Figure 5. The 19ηm ICAM-1 molecule consists of a 453 amino acid extracellular domain, a 24 amino acid transmembrane domain and a 28 amino acid cytoplasmic domain linked to the cytoskeleton. The extracellular domain has 8 N-linked glycosylation sites and 5 Ig-like domains supported by a β-pleated protein scaffold backbone. Though ligand binding is Ca^{2+} ion-dependent, structural conformation is not.

chromosome 19,[10,30] which combine to form an extracellular domain of 453 amino acids approximately 19 ηm in length.[30] The extracellular portion forms a hinged rod, with each Ig-like domain linked end to end in a β-pleated sheet.[30] There are also 8 sites for N-linked glycosylation of the Ig-domain, causing a variable molecular weight of 80-114 kD.[30] The molecule has a 24 amino acid transmembrane domain and a 28 residue cytoplasmic tail.[30] The cytoplasmic domain contains highly charged arginine and lysine amino acids and associates with the cytoskeletal protein α-actinin.[10,30,31]

ICAM-1 functions primarily as an adhesive ligand found on leukocytes, fibroblasts, epithelial and endothelial cells.[10] Each Ig-like domain is a potential binding site and thus supports promiscuous binding of ICAM-1. Glycosylation of the Ig-like domains is important in binding events as it can decrease the adhesive abilities of specific domains.[30,32] Complementary partners include non-cellular and cell surface proteins. Non-cellular ligands include fibrinogen, hyaluronan and Coxsackie A13 virus.[30] Adhesion to these soluble proteins allows cells to efficiently attach to the extracellular matrix within most tissues. Cellular ligands include the LFA-1 and Mac-1 integrins,[10,30] CD43,[33] MUC1,[34] and *Plasmodium falciparum*-infected red blood cells.[30] ICAM-1 adhesion to LFA-1 and Mac-1 occurs at the first and third amino terminal Ig-like domains respectively and is functionally important to T-cell stimulation and recruitment of inflammatory cells to the endothelium.[9,30] Integrin-mediated recruitment by ICAM-1 is dependent on prior rolling and integrin activation by cytokines.[22,23] Though conformation of ICAM-1 is not dependent on divalent cations, Mg^{2+} does enhance integrin ligation.[30,35,36] Binding to ICAM-1 mediates firm adhesion and arrest of cells on

endothelium with a high resistance to fluid flow shear stress.[23] Subsequently, it is speculated that association of ICAM-1 to cytoskeletal proteins mediates endothelial cell retraction and a permissive phenotype for migrating cells.[37]

Regulation of ICAM-1 expression is crucial to the specific recruitment of inflammatory cells. ICAM-1 is up-regulated by cytokines (eg. TNF-α and IL-1β), steroid hormones (eg. estradiol), and physical forces (eg. shear stress), while it is down-regulated by other substances including IL-4 and glucocorticoids.[30] The signaling pathways involved include second messengers, such as PKC, cAMP, and intracellular Ca^{2+}, which regulate transcription of ICAM-1 through activation or deactivation of the ICAM-1 promoter.[30,38,39] ICAM-1 may also be regulated through post-transcriptional means. For instance interferon-γ has been shown to increase ICAM-1 levels through stabilization of its mRNA.[30] Finally, ICAM-1 expression is also regulated through an unidentified cleavage mechanism which releases the extracellular domain to create a soluble ICAM-1.[30] This soluble form has been shown to dimerize non-covalently and has an increased affinity for the LFA-1 ligand in dimer form.[40,41] It is speculated that dimerization also occurs in the membrane bound form and regulates affinity and ligation to LFA-1.[40,41]

C) Step 3: Transmigration and Invasion

The integrin/IS ligands have also been demonstrated to mediate transmigration of immune cells through the vessel wall.[21,42] Both adhesion and migration can be prevented by antibody treatment against LFA-1 or VLA-4, or their respective endothelial ligands ICAM-1 and VCAM-1.[43] The endothelial IS molecules are inducible receptors capable of mediating intracellular signals. The association of ICAM-1 with the cytoskeleton

suggests that events post LFA-1 ligation may be involved in endothelial cell retraction and increased endothelial cell permeability.[31,37] To fully extravasate, cells must also traverse the basement membrane which is disrupted during neutrophil recruitment to the endothelium.[44,45] Intracellular signaling via the integrins and IS members mediate activation of matrix metalloproteinases and collagenases from immune and endothelial cells which are vital to dissolution of the basement membrane.[46-50] Integrin /IS ligation and signaling are thus vital to the completion of the transmigration process.

1.3.4 Theoretical Mechanisms of Tumor Cell Extravasation

The well characterized mechanisms of inflammatory cell extravasation clearly demonstrate the importance of adhesion molecules to this process. Although tumor cells express many of the same adhesion molecules, their exact extravasation process is still unknown.

There are two theories explaining tumor cell lodgement in distant capillary beds. The "passive occlusion" theory (Figure 6) suggests that tumor cells in circulation form aggregates or emboli with platelets and other intravascular tumor cells that eventually occlude capillaries nonspecifically.[51,52] Full extravasation would then be completed as static adhesive ligands would mediate migration.[51] The second theory argues that tumor cells extravasate through "active recruitment" otherwise considered the "extravasation cascade" (Figure 2). This involves an extravatory mechanism that would mimic that of inflammatory cells. Through binding of tumor and endothelial cell surface adhesion molecules, tumor cells would actively adhere to and migrate through endothelium at the distant site.[53,54] Only this mechanism of "active recruitment" would explain organ-specific metastasis which is commonly seen in cancer.[55,56] It is postulated

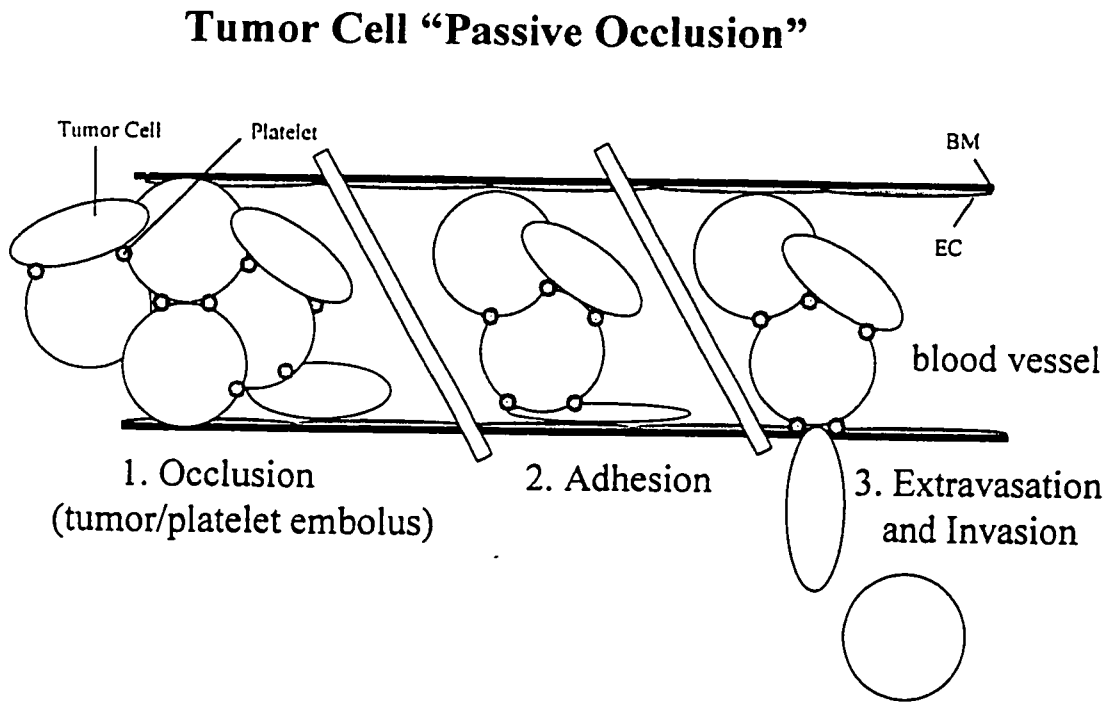


Figure 6. "Passive Occlusion" as a means of tumor cell lodgement and extravasation requires the formation of a tumor cell/platelet embolus. The large cellular clump would then occlude a capillary and allow for the static adhesion between tumor and endothelial ligands mediating extravasation.

that differing stromal constituents of various tissues would alter the endothelial adhesion molecule phenotype, causing specific recruitment of only those tumor cells with the appropriate ligands.[56] Though each mechanism employs distinct mechanisms of tumor cell lodgement, the requirement of adhesion exists in both cases to complete extravasation. Since tumors often express adhesion molecules similar to those on inflammatory cells, the mechanisms of tumor cell extravasation may mimic that of inflammatory cells.

1.3.5 Molecules of Tumor Cell Adhesion

Many of the molecules found on the surface of various tumor cells can serve as adhesive ligands in static and/or dynamic conditions.[31,55] These molecules can be separated into those which mediate "tethering" or those which mediate firm "arresting" adhesion of circulating cells to endothelium.

A) Molecules mediating "tethering"

There are a wide variety of surface molecules which mediate the transient rolling of tumor cells to endothelium. The most common are highly glycosylated and possess the carbohydrate selectin ligands in their glycosylated branches. This includes mucin proteins as well as CD44 and numerous other proteins which contain either sialylated or unidentified selectin ligands.[7,20,57] In most cases tumor cell rolling is then hypothesized to be a prerequisite for firm adhesion and arrest mediated by secondary adhesion molecule binding.[6]

B) Molecules mediating firm adhesion (arrest)

Molecules responsible for firm arrest of tumor cells on endothelium are similar to those described on inflammatory cells. These are primarily the β_1 integrins ($\alpha_4\beta_1$; VLA-4),[6,58,59] which bind the endothelial ligand, VCAM-1[6,58] although other IS members have also been implicated.[31] Though inflammatory cells have an absolute requirement of prerequisite rolling for firm arrest, certain tumor molecules have been proven to be more

efficient in their capacity to trigger cell arrest. The lung-derived endothelial cell adhesion molecule Lu-ECAM-1 can mediate arrest of cells on endothelium without primary adhesive ligands to mediate prerequisite rolling.[60]

Recent studies from our lab have implicated the breast tumor mucin molecule, MUC1, as a novel ligand for the IS member ICAM-1.[34,61] This interaction, if functional under physiological fluid flow conditions, may provide a molecular mechanism whereby tumor cells can initiate firm adhesion and transmigration by mimicking inflammatory cell interactions with ICAM-1.

1.4 The MUC1 Mucin Molecule

1.4.1 The Mucin Family

Mucins are a family of proteins primarily associated with glandular tissue. There are currently eight human and five rodent mucin genes which have been identified.[62] These genes are expressed throughout the body on the apical surface of cells in the pancreatic, salivary and breast glands as well as the lumenal surface of the bronchioles and gastric epithelium.[63] The function of these proteins has not been clearly defined on these surfaces despite the redundant structure common to all mucins.[64]

All mucins are specialized proteins which contain numerous O-linked glycosylations contributing to over 50% of their total molecular mass.[62] Each mucin gene contains a nucleotide sequence which is repeated multiple times in an end-to-end fashion, resulting in "tandem repeats" of the specific amino acid sequence in the extracellular portion of the protein.[62] Though each mucin has a low homology within the tandem repeat sequence, the repeat sequences all contain a high number of O-linked glycosylation sites (Ser and Thr amino acids)[62] such that as many as one in three amino acids may be glycosylated on each protein.[65] Qualitative alterations in mucin glycosylation occur during tumorigenesis which may be involved in tumor progression[63] and the promotion of tumor cell metastasis.[65] For example, in breast cancer the MUC1 mucin is altered and

these alterations have been shown to correlate with increased breast cancer progression and poor prognosis.[66,67]

1.4.2 Molecular Description of MUC1

MUC1 is the only type I transmembrane protein of the mucin family while all other members are secreted proteins (Figure 7).[68] MUC1 has a highly conserved 31 amino acid transmembrane domain and a conserved 69 amino acid cytoplasmic domain[62,69] which interacts with the actin cytoskeleton.[70] The extracellular domain of MUC1 consists mainly of a variable number (30 – 90) of tandem repeats, causing the entire length and molecular weight of the molecule to vary between 200 - 500 kD within a given population.[62,64] Each repeat consists of a heavily glycosylated 20 amino acid sequence.[68] The extracellular domain of MUC1 extends 200 - 500 ηm above the cell surface, far above the 10-30 ηm glycocalyx and other adhesion molecules on the cell surface.[64] The full MUC1 protein takes on a stable, rod like, conformation consisting of polyproline β-turn helices.[71] This conformation creates a knob-like immunogenic epitope (PDTRP) within the tandem repeat.[34,61] However, with less then 3 tandem repeats the protein loses its immunogenic knobs and reverts to a random coil that has been shown to be experimentally inert in function.[71,72] In 90% of breast cancers the numerous O-linked sugars on MUC1 are reduced exposing normally cryptic epitopes within the extracellular protein core (Figure 8).[34] The epitopes exposed during breast cancer-associated underglycosylation are possibly sites important for tumor progression.[34,61]

1.4.3 Anti-adhesive Function of MUC1

MUC1 is most often described as an anti-adhesive molecule through properties of both the cytoplasmic and extracellular domains. Due to the numerous O-glycosidic linkages, the extracellular domain of MUC1 carries a net negative charge which could cause repulsion of cells with similar glycosylation.[73,74] Furthermore, MUC1's length

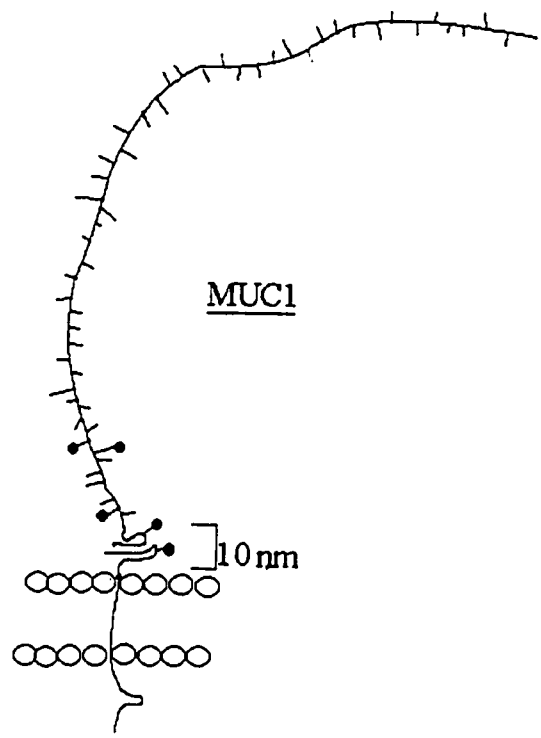
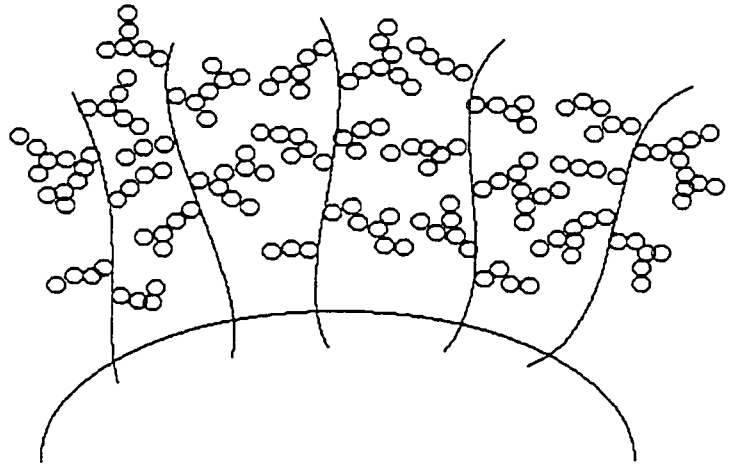


Figure 7. The MUC1 mucin molecule extends (500 ηm) high above the glycocalyx and other adhesion molecules (approx. 10 ηm). The highly glycosylated molecule maintains a rigid structure due to multiple Proline amino acids in the tandem repeat protein core. The extended conformation would then be readily available to interact with other cell molecules in an adhesive or anti-adhesive fashion.

Normal MUC1



Tumor MUC1

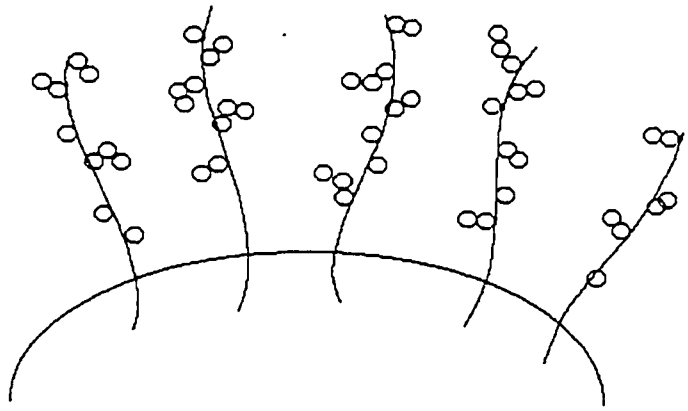


Figure 8. In breast carcinomas qualitative changes occur on the MUC1 mucin. Due to a loss of glycoslyltransferase function MUC1 is underglycosylated. This leads to exposure of the protein core, making epitopes available for possible adhesive processes.

and rigid structure is thought to shield adjacent, smaller adhesion molecules from interacting and binding their ligands.[73,74] Evidence in support of this non-specific anti-adhesive function is based on several transfection studies showing that overexpression of MUC1 is associated with impaired interactions with cytotoxic lymphocytes,[75-77] decreased integrin mediated cell-cell adhesion and decreased homotypic cellular aggregation.[64,73,74] As well, adhesion to extracellular matrix proteins, such as laminin, fibronectin and collagen is decreased with MUC1 expression as a result of these non-specific properties.[64,74,78] The cytoplasmic domain of MUC1 has also been implicated in anti-adhesion. It has recently been shown to compete with E-cadherin for β-catenin binding. The E-cadherin molecule mediates intercellular cell-cell adhesion of epithelial cells.[79,80] MUC1 overexpression in tumor cells sequesters the β-catenin molecule in the cytoplasm and causes the loss of the functional E-cadherin complexes needed for cell-cell adherence in epithelium (Figure 9).[79-81] This would allow the epithelial cell to migrate from its original site of growth and begin the invasion processes needed for metastases.

1.4.4 Arguments Against the Anti-Adhesive Role of MUC1

Advocates of the "Anti-adhesive" role of MUC1 argue that the normal function of MUC1 expressed on the apical aspect of glandular epithelium is to facilitate the formation and lumenal patency of the ductal system. Thus, the overexpression of MUC1 in breast cancers may result in a generalized loss of adhesion which facilitates the metastatic phenotype. However, MUC1 "knock out" mice have well formed, patent, glandular lumens,[67] while overexpression of MUC1 does not affect the *in vitro* growth or ability to adhere to stroma of normal breast epithelial cells with activated β_1 integrins.[74] It has also been suggested that the net negative charge created by multiple O-linked glycosylations of MUC1's extracellular domain is responsible for decreasing adhesion between cells. However, removal of sialic acid residues affected the anti-adhesive property only slightly.[73] Finally, in a report examining interactions between immune

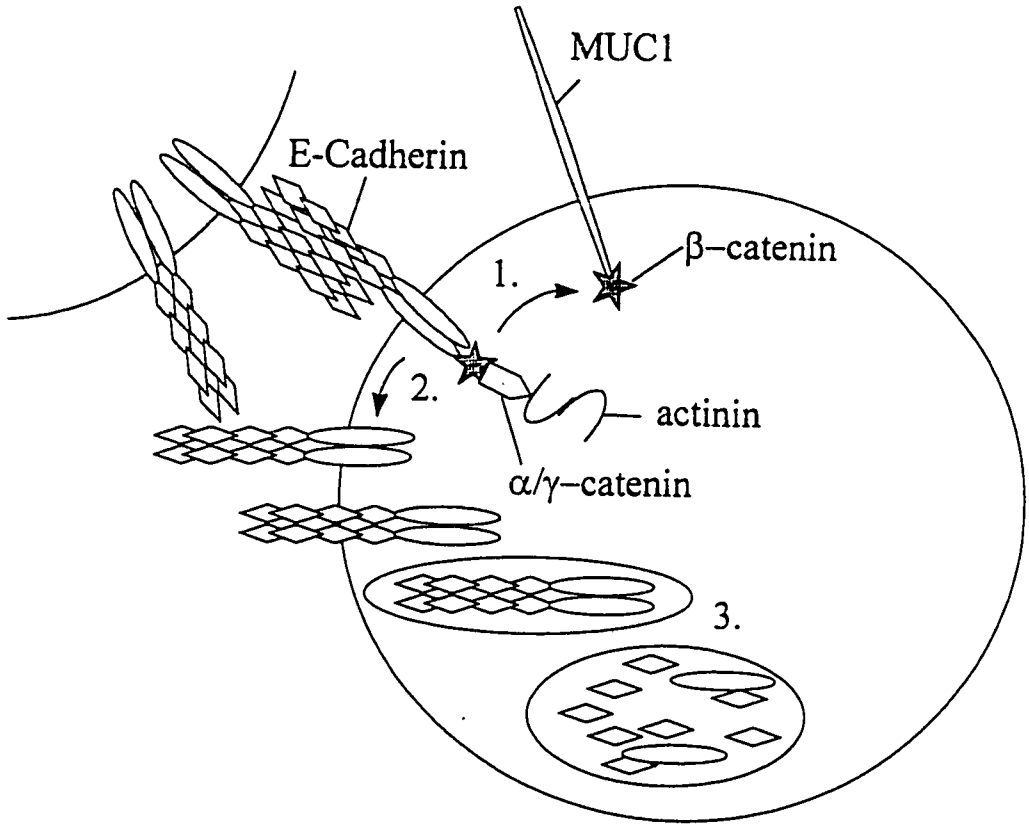


Figure 9. It is theorized that MUC1 may induce an invasive state by competing for the β-catenin molecule with E-cadherin, which is needed for adherens junction formation between epithelial cells. Briefly, MUC1 would effectively compete with E-cadherin for β-catenin (1). E-cadherin would then lose its cytoskeletal attachments which are normally maintained via β-catenin binding to α/γ-catenin and actinin (2). Loss of cytoskeletal attachment leads to the endocytosis and re-cycling of E-cadherin (3). Loss of E-cadherin surface expression would prevent homotypic binding, leading to a non-adherent and possibly invasive epithelial cell.

effector cells and MUC1 transfectants, it was found that lysis of the transfectants, but not the revertants (MUC1 negative cells), was decreased by antibodies to LFA-1 and ICAM-1.[76] Subsequently, Zhang et al. showed that soluble MUC1 was capable of specifically binding to effector cells.[77] Thus there are several inconsistencies in the putative role of MUC1 as strictly an anti-adhesive molecule.

1.4.5 Adhesive Function of MUC1

Recent publications describe MUC1 as a pro-adhesive molecule which is supported by three lines of evidence. First, MUC1carbohydrate residues, though decreased in cancer, contain selectin and lectin carbohydrate ligands involved in cell-cell adhesion.[57,82] These are the same ligands ($sLe^{a/x}$) that mediate "tethering" of inflammatory cells by adhesion to selectins.[21] It has been shown that mucins, including MUC1, can mediate similar adhesive events on tumor cells through these carbohydrate ligands.[57,82]

The second line of evidence supporting pro-adhesion is that MUC1 appears to be capable of intracellular signaling through mechanisms similar to those found on well established receptors.[69] MUC1 contains a tyrosine phosphorylation site on its cytoplasmic tail (Figure 10) that could contribute to regulation of multiple intracellular pathways.[69] One such pathway has already been identified as being mediated through the SH2 domain of Grb2, which binds a phosphorylated tyrosine containing sequence in the MUC1 cytoplasmic tail.[83] The Grb2 protein then binds SOS via a proline specific SH3 domain and recruits it to the membrane where it becomes involved in Ras activation, a known regulatory molecule of tumorigenesis.[83] Thus, it is possible that MUC1 ligation may cause phosphorylation of cytoplasmic sequences and lead to intracellular signaling important for migration and invasion.

Finally, the MUC1 protein core has been proven as a ligand for the ICAM-1 molecule in static assays.[34,61] There is a direct correlation between expression of each

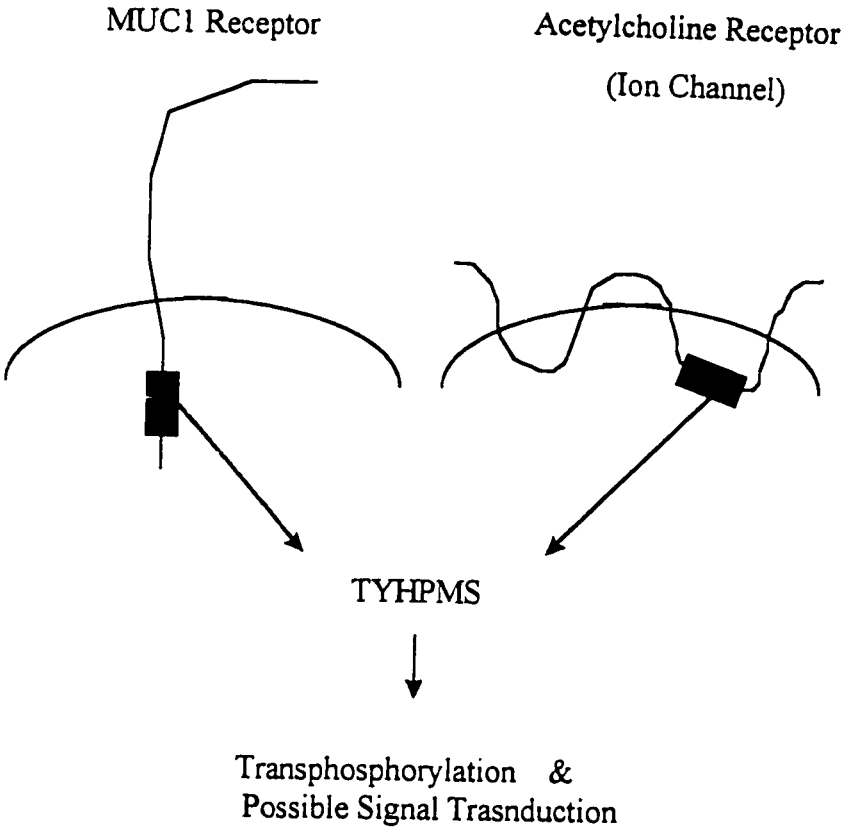


Figure 10. The MUC1 mucin contains a cytoplasmic signaling sequence known to exist and function in the acetylcholine receptor. The sequence, TYHPMS, is known to undergo transphosphorylation on the acetylcholine receptor and mediate signal transduction. It is then feasible that ligation of MUC1 by a ligand would trigger a similar function in tumor cells.

molecule and the ability of cells to adhere in static assays.[34] The binding is highly specific and efficient as MUC1-mediated tumor cell adhesion to purified ICAM-1 was inhibited by pretreatment with purified soluble MUC1.[34] The binding mechanism is similar to that of ICAM-1/integrin adhesion in two aspects. First, adhesion is dependent on the presence of divalent cations for high affinity binding.[84] Secondly, MUC1, like the integrins, has a specific conformational requirement for adhesion.[61] The adhesive interaction can only be inhibited by soluble MUC1 molecules consisting of 6 or more tandem repeats.[61] It is suggested that this is a result of the structural conformation of MUC1, which requires three or more tandem repeats to create a stable, rod-like conformation exposing the immunogenic "PDTRP" epitope on the protein core.[61,71] A similar conformational requirement must exist for MUC1 induction of T-cell anergy.[85-87] Thus, in breast cancer there is some evidence for a pro-adhesive role whereby the MUC1 molecule may enhance the metastatic ability of a tumor cell. This is one explanation for the *in vivo* observation that increased tumor MUC1 expression is associated with increased cancer stage and poor prognosis.[63,78,85,88] To date only static adhesion assays have been used to demonstrate the ability of MUC1 to bind ICAM-1. Further analysis under physiological fluid flow would determine the requirements needed for this interaction to occur within the circulation.

1.5 Dynamic Fluid Flow

1.5.1 Physiological Shear Stress

The shear stress forces in circulation play a major role in preventing cells within the flow of fluid from adhering to endothelium.[89] For a cell to successfully extravasate, the interactions between adhesion molecules on the stationary endothelial cell and the circulating cell must be stronger than the forces of fluid flow.

Shear stress in physiological fluid flow is directly dependent on both the fluid viscosity and velocity (more specifically volumetric flow rate).[90] Within a closed system,

such as a blood vessel, the viscosity of a flowing fluid is constant.[91] Thus, shear stress is directly dependent on the fluid velocity, which in turn is dependent on the distance from the vessel wall (Figure 11).[91] In an ideal system the velocity increases towards the center of fluid flow (known as laminar flow).[91] Shear stress is therefore greatest at the center of fluid flow, which is then the site of shear stress measurement.

At the site of most active cellular extravasation, the post capillary venules, shear stresses of 1-10 dyn/cm^2 have been demonstrated.[23] In artificial (*in vitro*) systems, leukocyte adhesive processes occur at less then 5 dyn/cm^2.[23,92] However, *in vivo* systems have demonstrated adhesion of cells at up to 30 dyn/cm^2.[92] Many theories have been proposed to explain this discrepancy. The most probable is that *in vitro* systems use endothelial cells cultures grown in static conditions. Upon shear stress exposure multiple phenotypic changes are induced on the endothelial cells, making the *in vitro* model different from the *in vivo* methods which are under continuous fluid flow.[92-94] Though *in vitro* adhesion assays are not ideal, they have been extremely useful in the progression of the current knowledge concerning the adhesive capabilities and requirements of specific ligands.

1.5.2 The *In Vitro* Parallel Plate Flow Chamber

In vitro dynamic adhesion assays are commonly used to determine the specific mechanisms involved in adhesion between a circulating cell and stationary endothelial cell. These assays were essential to the development of the adhesion cascade theory for the recruitment of inflammatory cells,[23,90,95] and are now being used to study tumor cell extravasation during metastasis.[6,60,61]

A common model used for *in vitro* adhesion assays is the parallel plate flow chamber which creates laminar fluid flow that can mimic physiological shear stresses. The chamber consists of a main deck which houses an entrance and exit slot for media and cells; a gasket separating the flow deck from the endothelial cells; and a glass slide

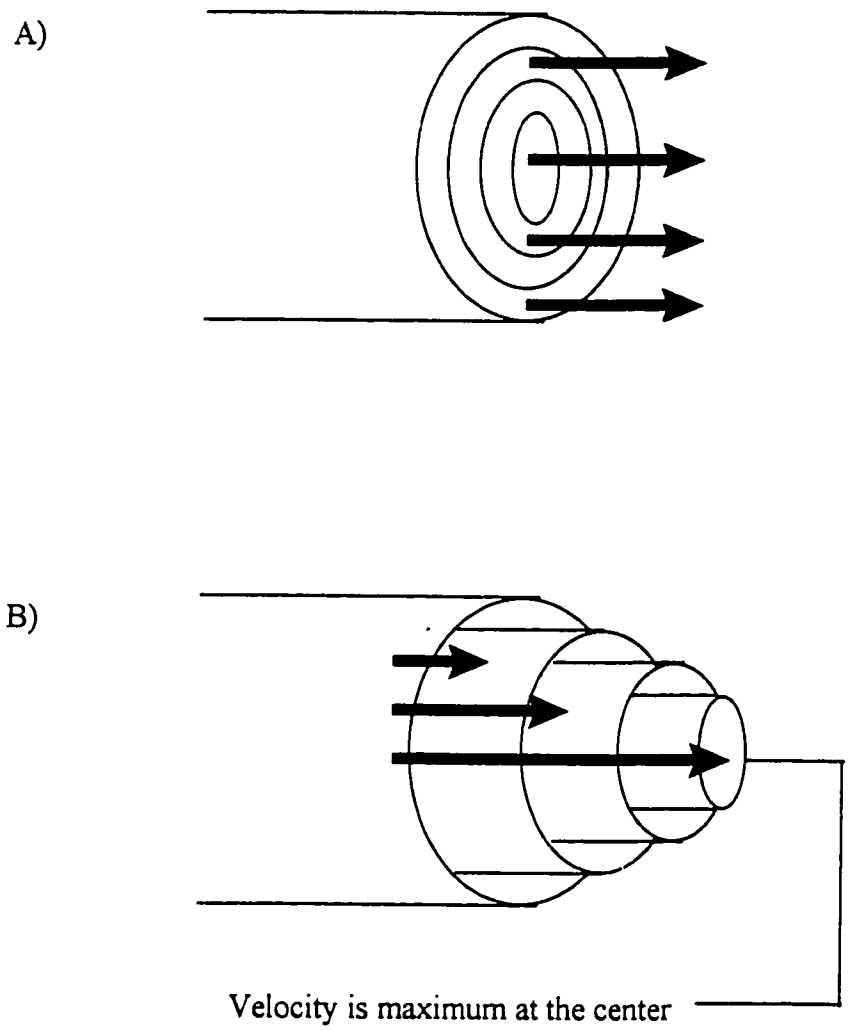


Figure 11. a) In an open system, without edges, fluid moves at a uniform velocity throughout. b) In a closed wall system, such as a blood vessel, fluid moves in a laminar fashion where layers of fluid move at an increasing velocity towards the center of the fluid. In an ideal system, fluid is then stationary at the wall.

with a confluent layer of cultured endothelial cells (Figure 12).[90] The shear stress created by the chamber has been mathematically described (Figure 13) and is directly proportional to both viscosity and volumetric flow rate, similar to *in vivo* conditions.[90] The chamber can thus be used to accurately reproduce physiological shear stress and effectively assay specific molecular requirements and adhesion of circulating cells to endothelium.

1.6 Purpose of the Study

This thesis examines the MUC1 molecule as a ligand and possible receptor capable of binding endothelial ICAM-1 under physiological shear stress. The ability of these molecules to recruit tumor cells to vascular endothelium under fluid flow advances the theory that both MUC1 and ICAM-1 are involved in tumor cell extravasation and metastasis. The specific involvement of the MUC1 molecule in this process suggests that MUC1 may provide a target for anti-metastatic therapy. This approach is very promising as the MUC1 target would reduce any adverse effects on immune cell extravasation and function, since inflammatory cells rarely express or utilize MUC1 during recruitment or extravasation.

Data presented in this thesis also addresses the molecular requirements needed for each molecule to bind in shear stress conditions. This information can then be compared and contrasted to the existing knowledge of ICAM-1 ligands such as the integrins (LFA-1, Mac-1) and the sialomucin (CD43). This will provide further insight into developing a target to specifically interfere with tumor cell metastasis.

1.7 Hypothesis

This thesis tests the hypothesis that tumor cell MUC1 can mediate cell recruitment by binding to endothelial ICAM-1 under fluid flow conditions. Previous data from our laboratory shows that breast cancer cells bind to endothelial monolayers in static

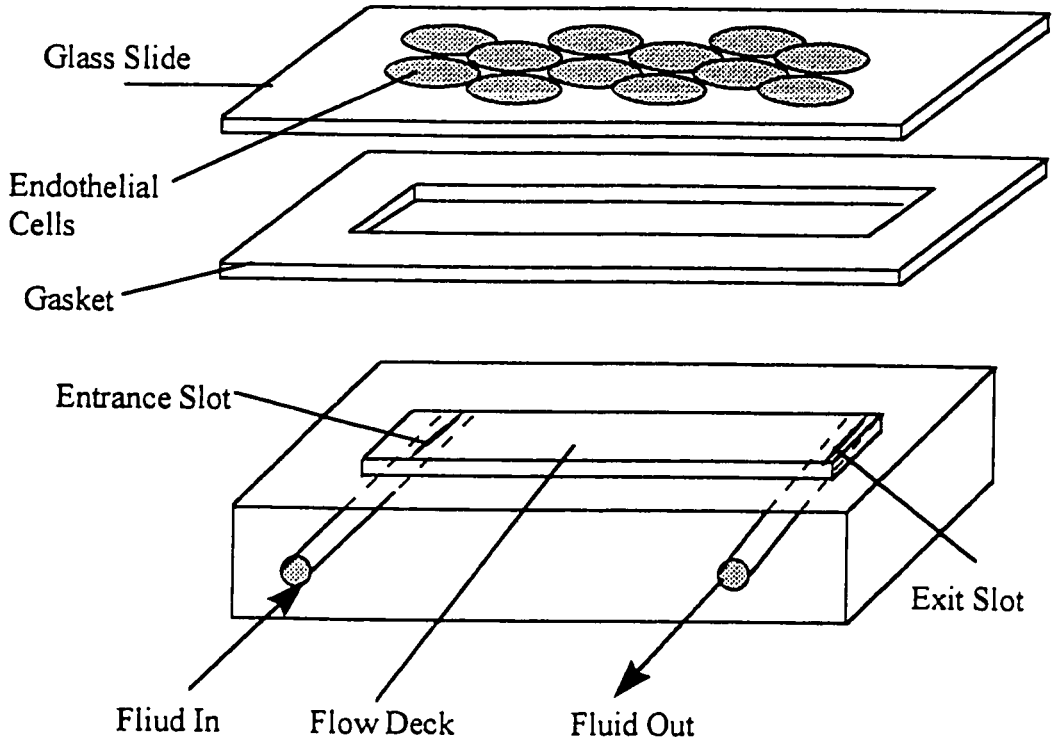


Figure 12. The parallel plate flow chamber allows for the reproduction of physiological shear stress in an *in vitro* model that can be readily manipulated. Cultured endothelial cells on a glass coverslip are separated from the flow deck by a gasket. Media containing target adherent tumor or inflammatory cells is injected into the model where they interact with endothelium. The circulating cells will either adhere to the endothelium and remain within the chamber or are non-adherent and are swept out of the model's exit slot.

$$\tau = \frac{3\mu Q}{2a^2 b}$$

τ = Wall Shear Stress
μ = Coefficient of Viscosity
Q = Volumetric Flow Rate
2a = Channel Height
b= Channel Width

Figure 13. The parallel plate flow chamber is mathematically described to produce physiological shear stress. For any given perfusing media all variables of the equation are constant, except for volumetric flow rate which can then be manipulated to accurately produce the desired shear stress.

adhesion assays specifically through the tumor MUC1 and endothelial ICAM-1 molecules.[34,61] Furthermore, this adhesion was characterized to be regulated by divalent cation presence, temperature, and MUC1 conformation.[61,84] Characterization of this binding in fluid flow conditions of physiological shear stress would further support the relevance of this interaction as a mechanism of active breast tumor cell metastasis.

Specific Objectives:

The primary experimental analyses will determine if MUC1-expressing tumor cells are specifically recruited to ICAM-1 expressing endothelium via a specific adhesive interaction between these two molecules. This will strengthen the hypotheses of: a) MUC1 as an adhesive ligand and b) active tumor cell extravasation within the circulation.

To achieve this goal a murine MUC1 transfectant breast carcinoma cell line (GzHi), and the parental cell line (410.4) will be used as the circulating target cells in dynamic fluid flow adhesion assays. The endothelial component (Ea-hy-926) consists of an immortalized hybrid of human umbilical vein endothelial cells (HUVECs) and a human lung carcinoma (A549) cell line. Together these cells are used in a parallel plate fluid flow adhesion assay. Subsequently, the major findings of these studies will be tested using a human breast carcinoma cell line and early passage primary cultures of human umbilical vein endothelial cells.

The following topics are addressed :

1) Correlation between MUC1/ICAM-1 surface expression and adhesion of tumor cells to the endothelial monolayer.
2) Specific inhibition of tumor cell recruitment using monoclonal antibodies to both MUC1 and ICAM-1 compared to an irrelevant antibody control.
3) Surface expression (on either cell line) of other adhesion molecules possibly involved in this adhesive cascade and known to be involved in immune cell adhesion.

4) Ability of the MUC1-expressing human breast carcinoma cell line (MCF-7) to adhere to HUVECs under physiological shear stress specifically through the MUC1/ICAM-1 interaction.

Secondary experimental analyses discussed in this thesis will address the specific molecular requirements of both MUC1 and ICAM-1 to mediate tumor cell adhesion under physiological shear stress.

The following topics are addressed :

1) ICAM-1 requirements for adhesion.
2) MUC1 requirements for adhesion.
3) Effect of varied shear stress rates on adhesion.

Chapter 2

METHODS AND RESULTS

2.1 Correlation of MUC1/ICAM-1 Expression to Tumor Cell/Endothelium Adhesion During Physiological Shear Stress

2.1.1 MUC1 Expression of Murine Breast Carcinoma (GzHi/410.4)

Objective:

To phenotype the murine breast carcinoma parental 410.4 and transfectant GzHi cell lines for human MUC1 expression. Major surface antigens were also phenotyped on each cell line to control for inadvertent selection of a sub population.

Materials and Methods:

Cells

Murine breast carcinoma cell lines (410.4 and GzHi) were generous gifts from Biomira Inc. (Edmonton, Alberta). The cells were maintained in RPMI 1640 (GIBCO Laboratories; Burlington, Ontario) supplemented with 10 % (v/v) fetal calf serum (FCS)(GIBCO), 2mM L-glutamine (GIBCO), 100 U/ml penicillin (GIBCO) and 100 μg/ml streptomycin (GIBCO).

mAbs

Monoclonal anti-MUC1 (B27.29) was a gift from Biomira Inc.; monoclonal anti-CD43 (MT1) and anti-Vimentin (V9) were donated by Laith Dabbagh (Cross Cancer Institute; Edmonton, Alberta); murine specific anti-$H2K^d$ (34-5-8S) and anti-$H2D^d$ (SF1.111) were the generous gift of Dr. K. Kane (University of Alberta: Edmonton, Alberta); monoclonal anti-ICAM-1 (18E3D) was a gift from Dr. M. Gallatin (ICOS

Corp.; Bothell, Washington); and monoclonal murine cross reactive anti-E-cadherin (DECMA) was a gift of Dr. M. Pasdar (University of Alberta; Edmonton, Alberta).

Flow Cytometry (2-step Indirect Method)

Cultured cells were washed in PBS and harvested using 0.05% (w/v) trypsin with 0.53 ml EDTA (EDTA-trypsin)(Sigma; Mississauga, Ontario). The cells were suspended in supplemented RPMI 1640 to quench the trypsin reaction, and centrifuged at 1200 rpm for 5 min. The supernatants were decanted and the cells were resuspended in PBS at approximately 1 x 10^6 cells/ml. Cell suspensions were then stained using a standard indirect procedure. 100 µl of primary monoclonal antibodies were added to cells at optimal concentrations (Table 1) and incubated for 45 min on ice. Cells were then washed with PBS, centrifuged and the supernatants discarded. 50 µl of secondary Phycoerythrin (PE)-labeled goat anti-mouse antibody (Southern Biotechnology Associates: Birmingham, Alabama) was then added to the cell suspensions for 30 min on ice, in the dark. After a final wash, cells were resuspended in PBS and analyzed by FACScan (Becton Dickinson; Franklin Lakes, New Jersey). Analysis was performed within 2 h of staining and used 10000 cells/analysis. Phenotyping was performed in triplicate. Gates were set beyond the fluorescent intensity of the negative control samples.

Results:

a) GzHi/410.4 MUC1 expression

Table 1. Monoclonal antibodies used in experiments

Ab Specificity	Clone	Method	Concentration (μg/ml)
MUC1	B27.29	FACScan	2.5
		Adhesion Blockade	2.5
		Immunoblot	0.5
Vimentin	V9	FACScan	1/20; 100 μl/test
H2-K^d	34-5-8S	FACScan	23
H2-D^d	SF1.111	FACScan	100 μl neat (supernatant)
ICAM-1	18E3D	FACScan	50
		ELISA	0.05-0.02
		Adhesion Blockade	50
	164B	FACScan	50
		Immunostain	50
		Adhesion Blockade	50
	164I	Adhesion Blockade	50
	Leu 54	FACScan	20 μl neat
VCAM-1	4B9	FACScan	6
E-cadherin	DECMA	FACScan	30 μl neat (supernatant)
CD31	JC/70A	FACScan	20
		Immunostain	20
		Adhesion Blockade	20
CD43	MT1	FACScan	100 μl neat (supernatant)
E-selectin	1.2B6	FACScan	33
P-selectin	AK-6	FACScan	20
LFA-1	IOT-16	FACScan	20
Mac-1	Leu-15	FACScan	20
VLA-4	H2/1	FACScan	2
sLe^x	CSLEX	FACScan	20
sLe^a	B67.4	FACScan	20

Table 1. (cont.) Monoclonal antibodies used in experiments

Ab Specificity	Clone	Method	Concentration (μg/ml)
CD34	8G12	Immunostain	100 μl neat
FVIII	F8/86	Immunostain	1/20; 100 μl/test
LCA	2B11, pD7/26/1	Immunostain	22.5
HRP-GAM	pooled	ELISA	1/5000-1/20000; 100μl/test
HRP-GAM	pooled	Immunostain	50
PE-GAM	pooled	FACScan	2
Biotin-GAM	pooled	Immunoblot	1/10000; 10ml/test

Table 1. List of monoclonal antibodies and concentrations used for various experimental procedures.

Both 410.4 and GzHi cells displayed comparable levels of all surface antigens tested, indicating that transfection and the subsequent selection of GzHi cells had no inadvertent selection bias on the expression of other potential surface adhesion molecules (Figure 14). The two cell lines differed significantly in human MUC1 expression with the 410.4 cells showing background (<5 %) levels while 55 % of the GzHi cells were positive (Figures 15 and 16).

2.1.2 ICAM-1 Expression of Ea-hy-926

Objective:

To phenotype the Ea-hy-926 endothelial-lung carcinoma hybrid cell line in resting and cytokine-stimulated conditions for ICAM-1 expression.

Materials and Methods:

Cells

The Ea-hy-926 cell line was the kind gift of Dr. C. J. S. Edgell (University of North Carolina; Chapel Hill, North Carolina). These cells are well described[96,97] and derive from somatic cell hybridization of a human umbilical vein endothelial cell (HUVEC) and a human lung carcinoma cell line (A549). Ea-hy-926 cells were cultured in supplemented RPMI 1640 containing 10 % FCS, 2mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

mAbs

The PE conjugated monoclonal anti-ICAM-1 (Leu-54)(Becton Dickinson) and the

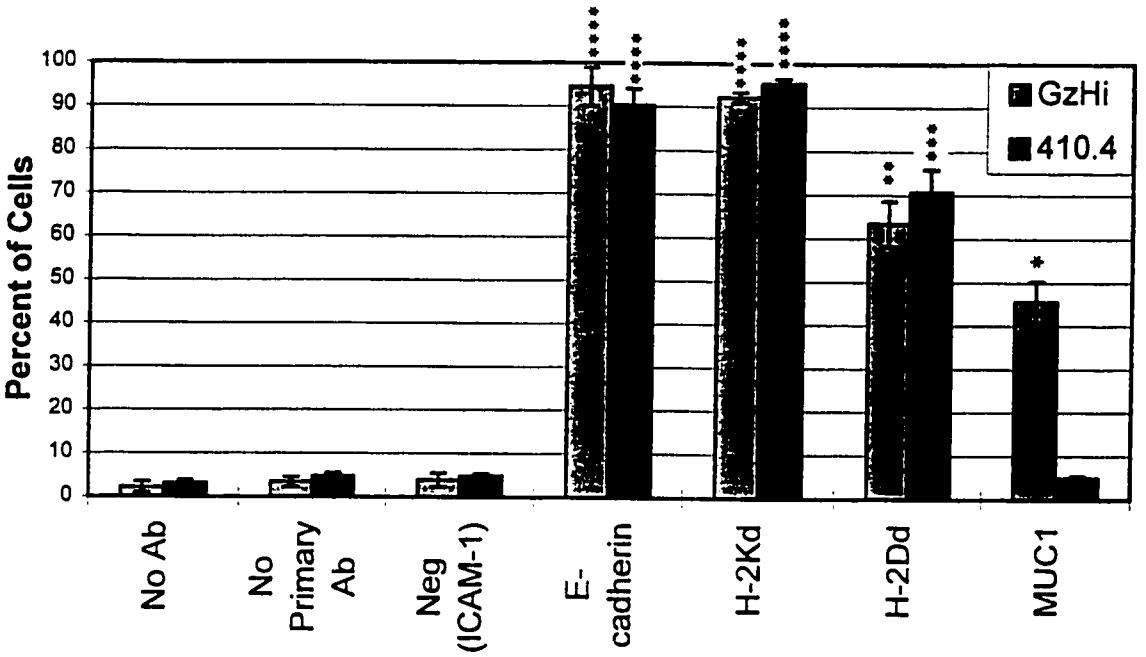
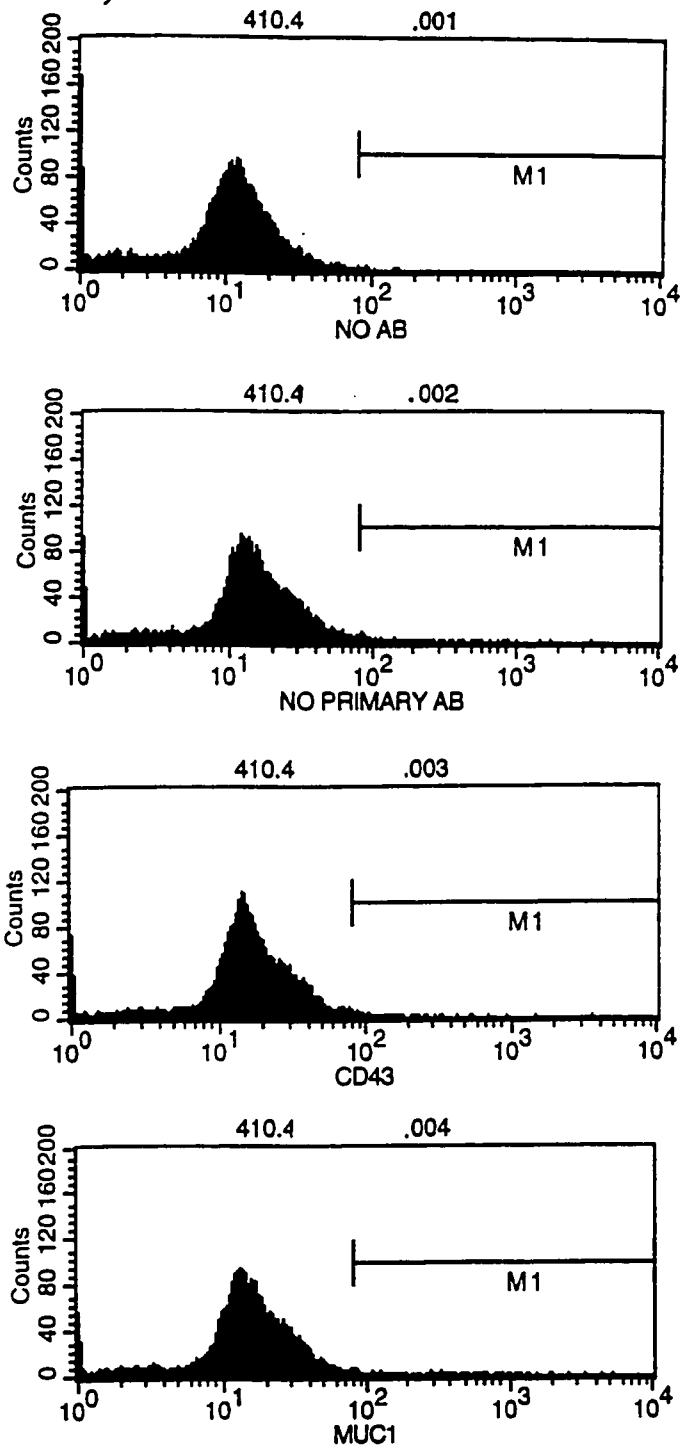


Figure 14. Surface expression of major surface antigens on GzHi and 410.4 tumor cells analyzed by flow cytometry. "*"-"****" represent distinct populations with statistical significance ($p<0.05$) between each as determined by Newman-Keuls analysis. Bar graph represents the mean +/- 1 s.d. of 3 repeats.

Figure 15.

A)



% Gated
100.00
0.12

% Gated
100.00
1.50

% Gated
100.00
0.88

% Gated
100.00
1.74

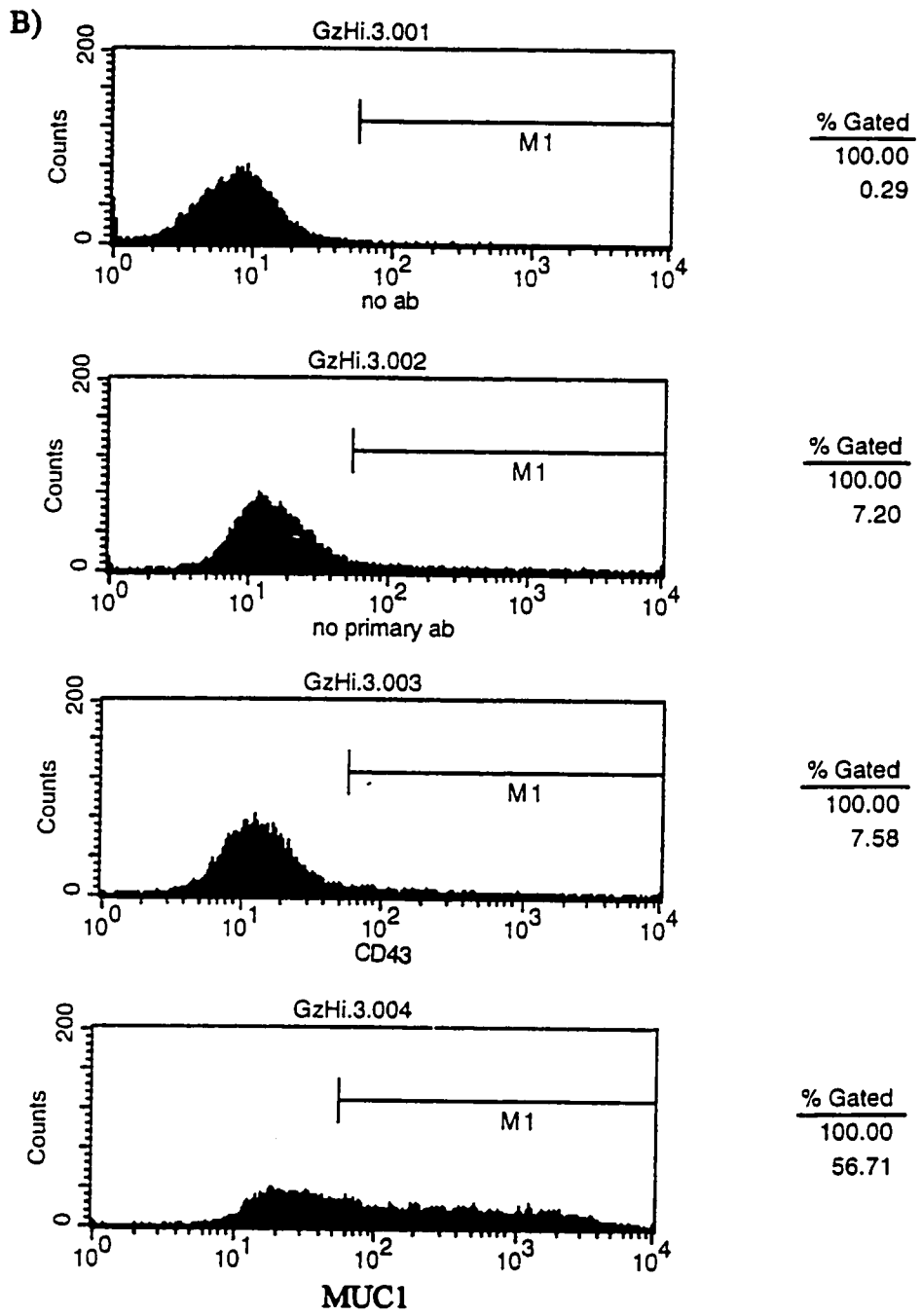


Figure 15. Histograms of GzHi/410.4 fluorescent intensity from FACScan analysis to determine MUC1 expression. Gates are set according to the negative controls. All subsequent flow cytometry FACScan analysis were performed using similar criteria.

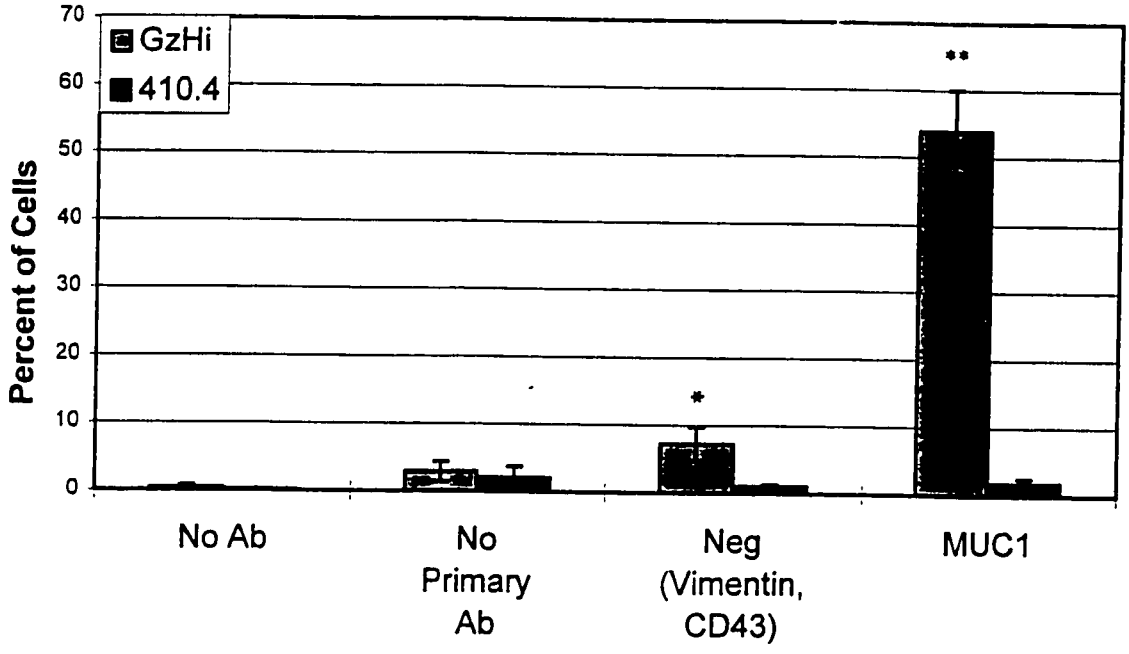


Figure 16. Surface expression of MUC1 on GzHi and 410.4 tumor cells as analyzed by flow cytometry. "*"-"**" represent distinct populations with statistical significance ($p<0.05$) between each as determined by Newman-Keuls analysis. Bar graph represents the mean +/- 1 s.d. of 3 repeats.

negative control, PE conjugated goat anti-mouse (Southern Biotechnology Associates), were used at optimal concentrations (Table 1) in the phenotyping procedure.

Endothelial Cell Cytokine Stimulation

Ea-hy-926 cells in passage 3-5 were stimulated with 20 U/ml of both TNF-α (PharMingen; Mississauga, Ontario) and IL-1 β (PharMingen) in supplemented RPMI 1640. 1ml of cytokine solution was added to culture flasks and incubated for 24 h at 37^{0}C. A flask containing 1 ml of supplemented RPMI 1640 without the addition of cytokines was used as a negative control. Cells from each flask were then harvested as outlined in the previous section and analyzed by direct FACScan analysis.

Flow Cytometry (Direct Staining)

A 1 x 10^6 cells/ml suspension (100 µl) was treated with 100 µl of PE-labeled antibody for 45 minutes on ice in the dark. After incubation cells were washed in PBS, centrifuged, the supernatant decanted and the cells resuspended in 300 µl PBS. Cells were immediately analyzed by FACScan at 10000 cells/ analysis. All experiments were performed in triplicate.

Results:

Ea-hy-926 cells stimulated with cytokines for 24 h had significantly increased levels of ICAM-1 expression. All negative controls and untreated Ea-hy-926 cells had minimal levels of fluorescence detected on their surfaces (Figure 17).

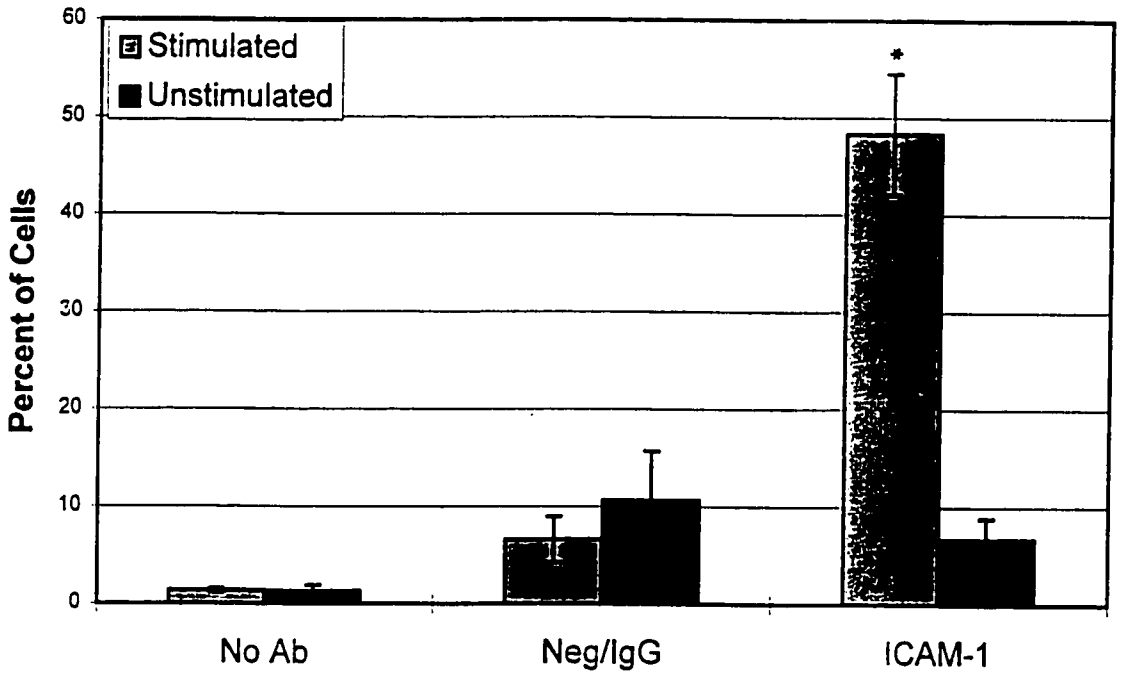


Figure 17. Differential ICAM-1 expression on Ea-hy-926 cells with or without 24h cytokine stimulation; analyzed by flow cytometry. "*" represents statistical significance determined by Newman-Keuls analysis ($p<0.05$). Bar graph represents the mean +/- 1 s.d. of 3 repeats.

2.1.3 Adhesion of GzHi/410.4 to Ea-hy-926 (+/- cytokine stimulation)

Objective:

To determine if the ability of tumor cells to bind endothelium in fluid flow is enhanced by the increased expression of MUC1 on tumor cells and/or ICAM-1 on endothelial cells. All adhesion experiments were conducted under fluid flow conditions of physiological shear stress.

Material and Methods:

Cells

Tumor cell lines (410.4 and GzHi) were cultured using supplemented RPMI and used as the cell population in circulation during the fluid flow adhesion assays. The Ea-hy-926 cell line were grown in supplemented RPMI and used as the stationary endothelial component in the assays.

Tumor cell/endothelial cell fluid flow adhesion assay

Monolayers of Ea-hy-926 cells were prepared on glass slides (Surgipath; Winnipeg, Manitoba) during passage 1-9. Cells were harvested from culture flasks using 300 μl of 0.05% EDTA-trypsin and then quenched and suspended in 500 μl of supplemented RPMI 1640. 700 μl of the cell suspension (approximately 1 x 10^6 cells/ml) was then coated onto two glass slides and cultured in supplemented RPMI 1640 until confluent (4-13 days). 24 hours prior to use in adhesion assays the media of confluent endothelial monolayers was exchanged for media with or without the addition of TNF-α/IL-1β (20 U/ml). The endothelial monolayers were then loaded into the parallel plate

flow chamber (gift of Dr. M.B. Lawrence; Harvard Medical School; Boston, Massachusetts) as shown in Figure 12. The endothelial monolayer is separated from the flow deck by a 250 μm Silastic gasket (Sil-Tec Technical Products Inc.; Decatur, Georgia).

410.4 and GzHi cells were removed from flasks using a non-enzymatic cell dissociation solution (CDS)(Sigma). After quenching the reaction with supplemented RPMI, cells were centrifuged at 1200 rpm for 5 minutes and the media was decanted. Two mls of RPMI with 4 μl of $2^{1},7^{1}$-bis-(carboxyethyl)-5(and 6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM; Molecular Probes, Eugene, Oregon), a fluorescent indicator derivative, was added to each flask and incubated for 45-60 minutes at 37^{0}C. The non-fluorescent, uncharged AM ester is internalized by the cells and cleaved by nonspecific esterases creating a charged free acid fluorescent form that cannot readily leave the cell. After incubation with the BCECF-AM the cells were centrifuged at 1200 rpm for 5 minutes and resuspended in "flow media" consisting of RPMI containing 3.75 mg/ml bovine serum albumin (BSA)(Sigma), 1.2mM Ca^{2+} and 1.0 mM Mg^{2+}.[23,90] The cell concentration was adjusted to a concentration between 0.3 - 3.5 x 10^{5} cells/ml prior to use.[90]

The BCECF-labeled tumor cell suspension was then perfused into the flow chamber over the Ea-hy-926 monolayer in a 37^{0}C, 5 % (v/v)CO_2 incubator. Using parameters described by Lawrence et al.[23,90] the volumetric flow rate was adjusted using a multi-syringe Harvard '90' syringe pump (Harvard Apparatus; Saint Laurent, Quebec) to create a shear stress of 1 dyn/cm^2. This shear stress is characteristic for that exerted in the

post-capillary vessels, the site of active extravasation.[95] After 30 minutes of fluid flow the flow chamber was flushed with 1 ml of non-cellular flow media, removing non-adherent tumor cells. One ml of EDTA-trypsin was then injected into the chamber and incubated for 10 minutes in the 37^{0}C, 5 % CO_2 incubator. All endothelial cells and any remaining 410.4 or GzHi cells were subsequently washed from the flow chamber using 5 mls PBS, collected and concentrated by centrifugation for 5 minutes at 1200 rpm and 4^{0}C. The cells were then resuspended in 750 µl of PBS and the amount of adherent GzHi or 410.4 cells was quantitated by detecting the BCECF fluorescent label present within the final wash. Since the chamber had been flushed twice prior to collection of the final wash, any fluorescence represented target cells which had bound to the endothelial monolayer. The fluorescent signal was quantified using a SPEX fluorimeter (Laboratory of Dr. A. Shaw; Cross Cancer Institute; Edmonton, Alberta), and compared with other signals detected. All adhesion experiments were completed in triplicate. The amount of adhesion was determined by the fluorescent signal detected after collection of labeled adherent tumor cells and expressed relative to a control which was run simultaneously.

Results:

GzHi cells were found to adhere to ICAM-1-expressing endothelium (cytokine stimulated) significantly more than the wildtype 410.4 cells (Figure 18). This increased adherence was seen only in the presence of cytokine-stimulated endothelium (high ICAM-1 expression)(Figure 19). These results demonstrate that a positive correlation exists between MUC1/ICAM-1 expression and tumor cell adhesion to endothelial monolayers under physiological shear stress.

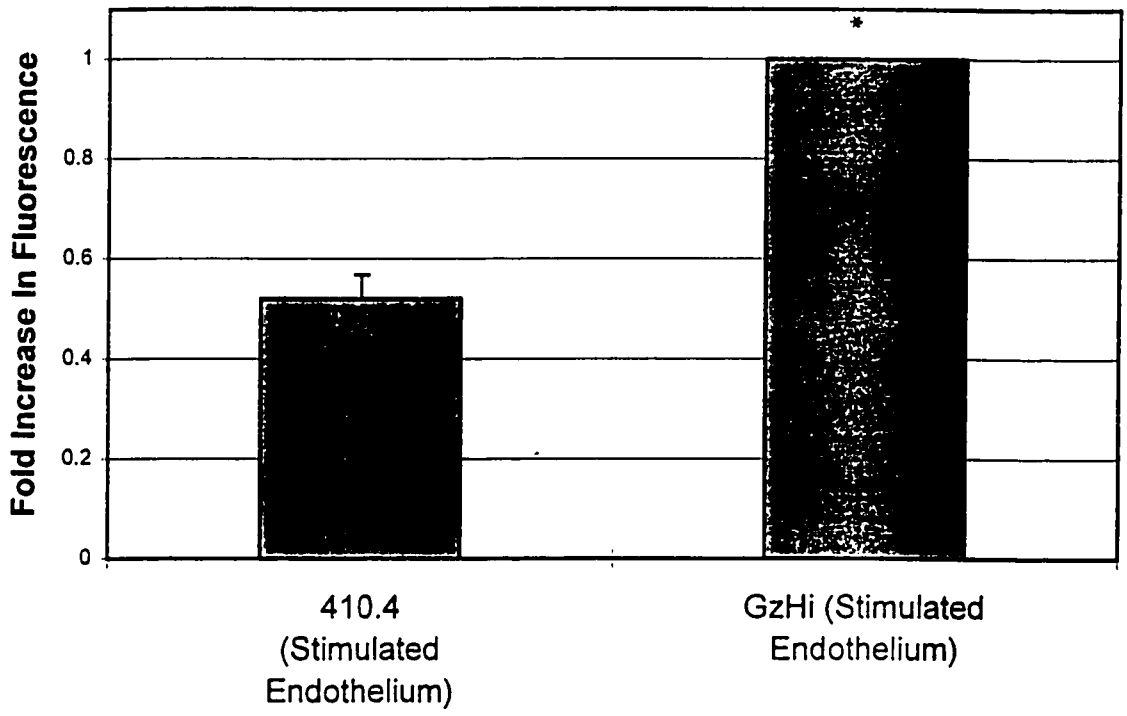


Figure 18. Adherence of GzHi and 410.4 tumor cells to cytokine stimulated Ea-hy-926 monolayers at physiological shear stress of 1.0 dyn/cm^2. "*" represents statistical significance determined by Newman-Keuls analysis ($p<0.05$). Bar graph represents the mean +/- 1 s.d. of 3 repeats.

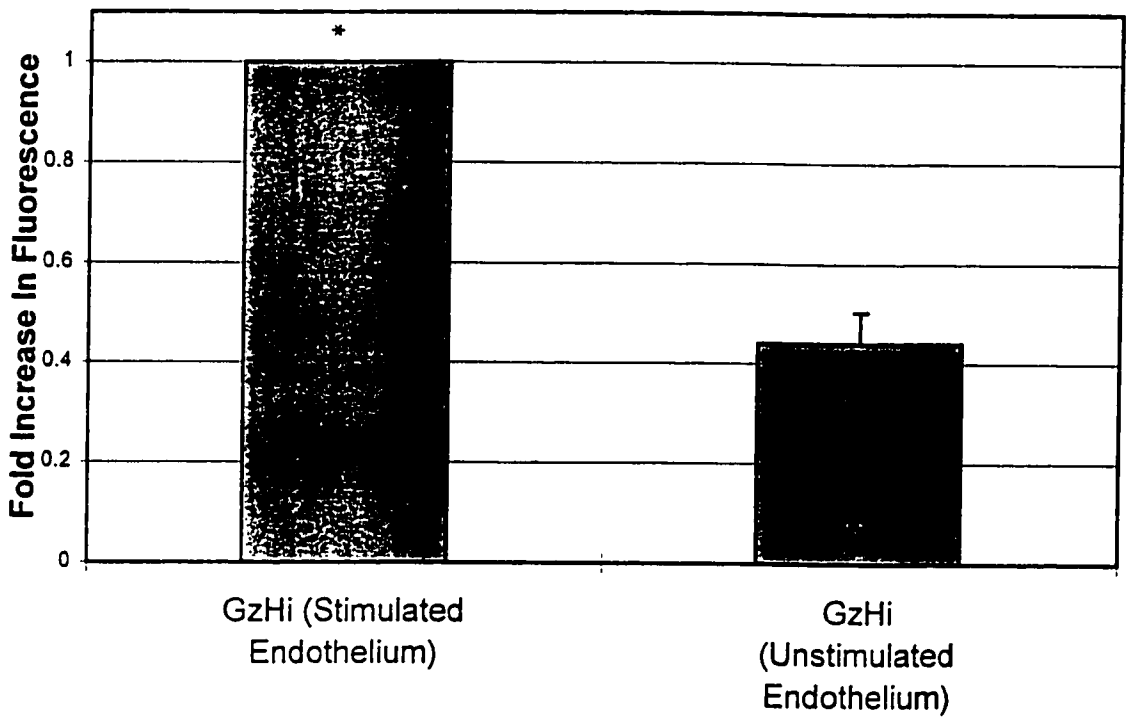


Figure 19. Recruitment of GzHi tumor cells to Ea-hy-926 endothelium with and without 24h cytokine stimulation under physiological shear stress. "*" represents statistical significance determined by Newman-Keuls analysis ($p<0.05$). Bar graph represents the mean +/- 1 s.d. of 3 repeats.

2.1.4 Summary of 2.1

The murine breast carcinoma cell line GzHi is the human MUC1 transfectant derivative of 410.4. Phenotypic analyses in this section show that MUC1 expression on GzHi cells is significantly greater than that of the parental 410.4 while other major surface antigens, such as MHC I and E-cadherin, are not altered between the two cell lines. This suggests that the two cell lines are phenotypically similar except for the presence of human MUC1 and that there has been no inadvertent selection of a distinct sub-clone.

The Ea-hy-926 cell line has differential ICAM-1 expression upon stimulation with cytokines (TNF-α and IL-1 β). After 24 h exposure to cytokines the Ea-hy-926 cells have significantly increased levels of ICAM-1 surface expression.

A positive correlation was found to exist between tumor cell/endothelial cell adhesion in fluid flow and MUC1/ICAM-1 expression. Significantly more GzHi cells were recruited to cytokine-stimulated endothelium than 410.4 cells. This demonstrates the correlation between increased tumor MUC1 expression and increased adhesion to endothelium. A relationship between ICAM-1 expression and adhesion was also evident as cytokine-stimulated endothelial cells (high ICAM-1 expression) recruited more tumor cells than unstimulated monolayers (low ICAM-1 expression).

2.2 MUC1/ICAM-1 Role In Tumor Cell Adhesion During Physiological Shear Stress

2.2.1 Antibody Inhibition of Adhesion

Objective:

This assay was performed to determine if the MUC1 and ICAM-1 molecules were functionally responsible for recruitment of GzHi cells to cytokine-stimulated Ea-hy-926 monolayers. Monoclonal antibodies were used to inhibit the tumor cell adhesion and implicate each molecule as a specific mediator of the adhesive process.

Material and Methods:

Cells

GzHi cells were cultured and used as the tumor cells which were perfused though the parallel plate flow chamber containing confluent Ea-hy-926 endothelial monolayers.

mAbs

The monoclonal B27.29 antibody, which binds a 6 amino acid peptide sequence in the tandem repeat of the MUC1 mucin protein core was used to block MUC1 in adhesion experiments. This antibody was chosen since previous results had shown that the epitope recognized by this antibody was responsible for binding ICAM-1.[34,61] A cocktail of three functional ICAM-1 blocking antibodies were used to prevent ICAM-1 interaction during fluid flow assays. All three ICAM-1 antibodies (164B, 18E3D, and 164I) were gifts of ICOS Corp. and are of the IgG_1 isotype. The 164B and 164I antibodies block the LFA-1/ICAM-1 interaction (correspondence with ICOS Corp.), while 18E3D has been shown to block the MUC1/ICAM-1 interaction in static assays.[84]

Anti-CD31 antibody (JC/70A)(Dimension Laboratories Inc.; Mississauga, Ontario), specific for the PECAM-1 molecule present on endothelial monolayers, was used as a negative inhibition control. Table 1 lists the concentrations of each antibody used.

Antibody Inhibition of Fluid Flow Adhesion Assay.

GzHi tumor cells were perfused over cytokine-stimulated Ea-hy-926 endothelial cells in a parallel plate fluid flow adhesion assay. Prior to MUC1 blockade experiments, GzHi cells were harvested, labeled with BCECF and divided into two aliquots. One aliquot was treated with the anti-MUC1, B27.29 antibody (60 μg/ml), for 45 min at 37^{0}C, while the other was mock treated with PBS for 45 min at 37^{0}C. After incubation and centrifugation the number of cells in each aliquot was quantitated using a ZBI Coulter counter (laboratory of Dr. A. Shaw). Each aliquot was then resuspended in "flow media" at identical concentrations according to the parameters previously described (2.1.3). Each cell population (+/- B27.29 antibody) was then infused into the parallel plate flow chamber at 1 dyn/cm^2 over cytokine-stimulated endothelial monolayers.

For ICAM-1 blockade and CD31 control experiments confluent Ea-hy-926 monolayers, stimulated with cytokines (TNF-α and IL-1β) for 24 hrs, were washed (x4) in PBS containing 1.0 mM Ca^{2+} and 0.5 mM Mg^{2+}. Monolayers were then treated with the anti-ICAM-1 cocktail or the CD31 antibody for 30 minutes at 37^{0}C. After incubation the antibody-treated endothelial monolayers were washed in PBS before being loaded into the parallel plate chamber and exposed to shear stress adhesion assays. Adhesion was based on the fluorescent signal of the final wash after washing and incubating with

EDTA-trypsin for 10 min (2.1.3).

Results:

Incubation of GzHi cells with the anti-MUC1 antibody (B27.29) significantly decreased recruitment of the tumor cells to cytokine-treated endothelium (Figure 20). The level of adhesion is almost identical to that of non-MUC1 expressing 410.4 cells.

The anti-ICAM-1 cocktail also decreased adhesion of GzHi cells to the endothelium (Figure 21). This inhibition was specific for ICAM-1 binding as treatment with the CD31 monoclonal antibody had no effect on adhesion despite the presence of CD31 on endothelium (Figure 21).

Thus, the adhesion of GzHi cells to Ea-hy-926 endothelium is specifically mediated by the MUC1 and ICAM-1 molecules respectively.

2.2.2 Adhesion Molecule Phenotype of GzHi and Ea-hy-926

Objective:

To determine if the GzHi and Ea-hy-926 cell lines express other surface ligands involved in normal immune cell recruitment. As outlined in the introduction inflammatory cells must first roll via selectins and their carbohydrate ligands prior to firm arrest mediated by ICAM-1 binding the integrins, LFA-1 and Mac-1.

Materials and Methods:

Cells

GzHi and Ea-hy-926 cells were cultured in supplemented RPMI. Ea-hy-926 cells were stimulated with TNF-α/IL-1β for 24 hrs.

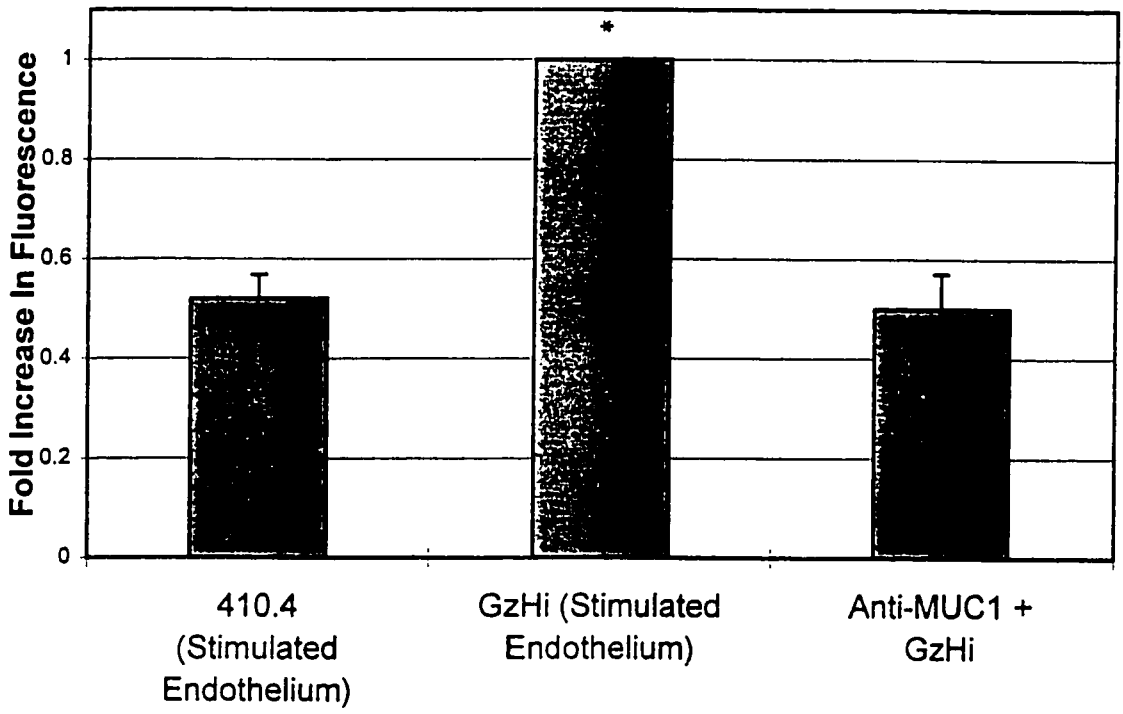


Figure 20. GzHi tumor cell adhesion (+/- anti-MUC1 pretreatment) to 24h cytokine stimulated Ea-hy-926 monolayers under physiological shear stress. "*" represents statistical significance determined by Newman-Keuls analysis ($p<0.05$). Bar graph represents the mean +/- 1 s.d. of 3 repeats.

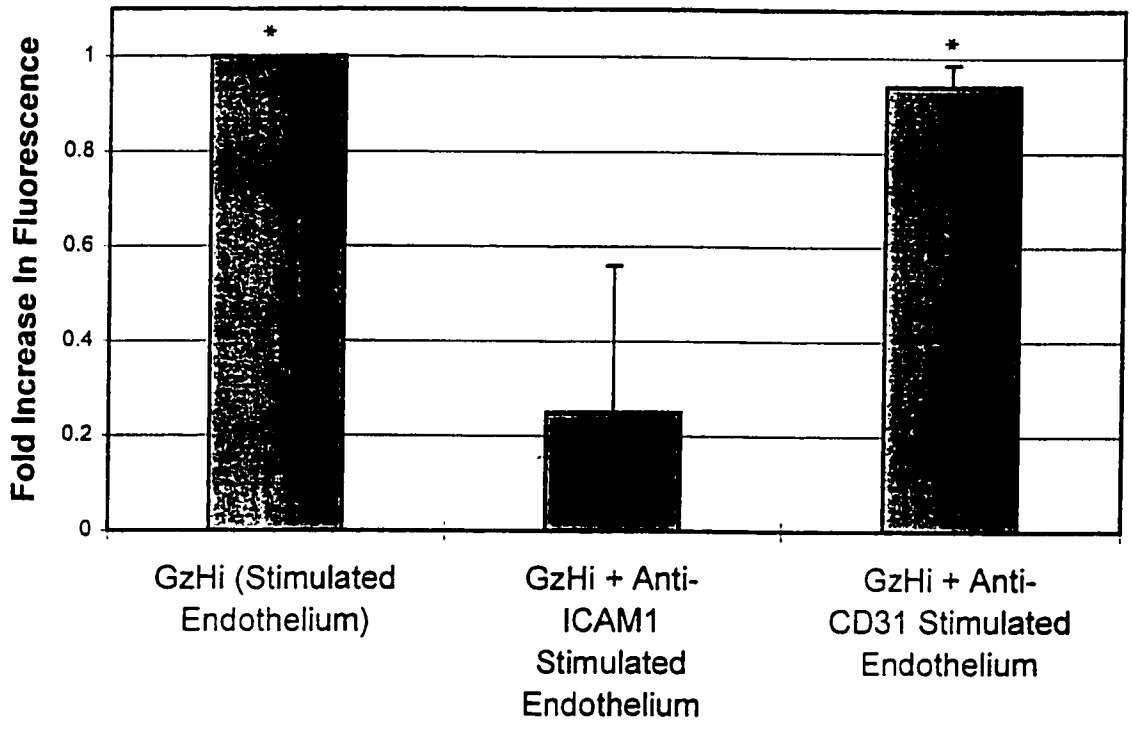


Figure 21. Adhesion of GzHi tumor cells to 24h cytokine stimulated Ea-hy-926 monolayers (+/- antibody pretreatment) under physiological shear stress. "*" represents statistical significance determined by Newman-Keuls analysis ($p<0.05$). Bar graph represents the mean +/- 1 s.d. of 3 repeats.

mAbs

Murine monoclonal antibodies specific for various surface adhesion molecules were used as the primary antibodies in a 2 step-indirect method for FACScan analysis. These include: anti-CD43 (MT1); anti-E-selectin (1.2B6)(Serotec Canada; Ontario); anti-P-selectin (AK-6)(Southern Biotechnology Associates); anti-LFA-1 (IOT-16), gift of Dr. L.M. Pilarski (Cross Cancer Institute; Edmonton, Alberta); anti-Mac-1 (Leu-15), gift of Dr. L.M. Pilarski; anti-VLA-4 (H2/1), gift of Dr. K. Patel (University of Calgary; Calgary, Alberta); anti-VCAM-1(4B9), gift of Dr.K.Patel; anti-sLe^x (CSLEX)(gift of Biomira Inc.); anti-sLe^a (B67.4)(gift of Biomira Inc.); anti-CD31 (JC/70A); anti-MUC1 (B27.29); and anti-ICAM-1 (18E3D). The secondary antibody for all tests was PE-labeled goat anti-mouse (Southern Biotechnology Associates).

Flow Cytometry (2-Step Indirect Method)

Secondary GAM-PE antibody was added after treatment with primary antibody and subsequent PBS washing. After a final PBS wash cells were analyzed by FACScan.

Results:

a) GzHi phenotype

These cells were negative for all tested adhesion molecules except for the MUC1 molecule (Figure 22). It should be noted however, that the antibodies used were raised against human antigens and thus murine cross reactivity is not guaranteed. This phenotype is similar to human epithelial cells except for the absence of the carbohydrate

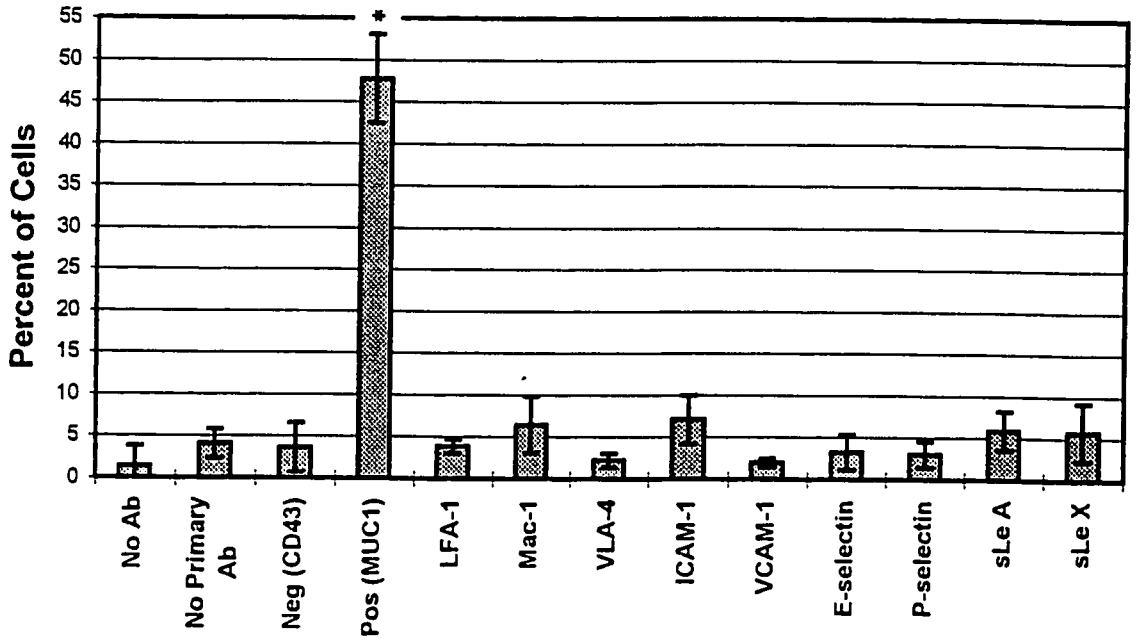


Figure 22. Surface adhesion molecule phenotype of GzHi cells analyzed by flow cytometry. "*" represents statistical significance determined by Newman-Keuls analysis ($p<0.05$). Bar graph represents the mean +/- 1 s.d. of 3 repeats.

selectin ligands residues due to the absence or impaired function of β_1-6GlcNAc transferases.[99]

b) Cytokine-stimulated Ea-hy-926 phenotype

These cells were negative for significant expression of selectins (E and P), integrins (LFA-1, Mac-1, VLA-4), and the IS member VCAM-1. Only sLe^a (a selectin ligand) and CD31 (PECAM-1) are present in significant amounts on cytokine-stimulated Ea-hy-926 (Figure 23).

2.2.3 Summary of 2.2

The MUC1 and ICAM-1 molecules were shown to be specifically responsible for adhesion of GzHi cells to cytokine-stimulated Ea-hy-926 monolayers in physiological shear stress. Monoclonal antibodies specific for both MUC1 and ICAM-1 diminished the adhesive interaction between these cells. Treatment with antibody specific for an endothelial cell surface molecule not involved in the adhesive process had no effect on the adhesion of GzHi to Ea-hy-926 thereby excluding non-specific steric hindrance of the antibody inhibition experiments.

Many other adhesion molecules are known to recruit inflammatory cells to endothelium through specific adhesion interactions. Phenotypic analysis shows that those adhesion ligand pairs involved with inflammatory cell adhesion are not present on the GzHi or stimulated Ea-hy-926. Thus, these molecules are likely not contributing to a tumor cell adhesion cascade involving MUC1/ICAM-1 binding.

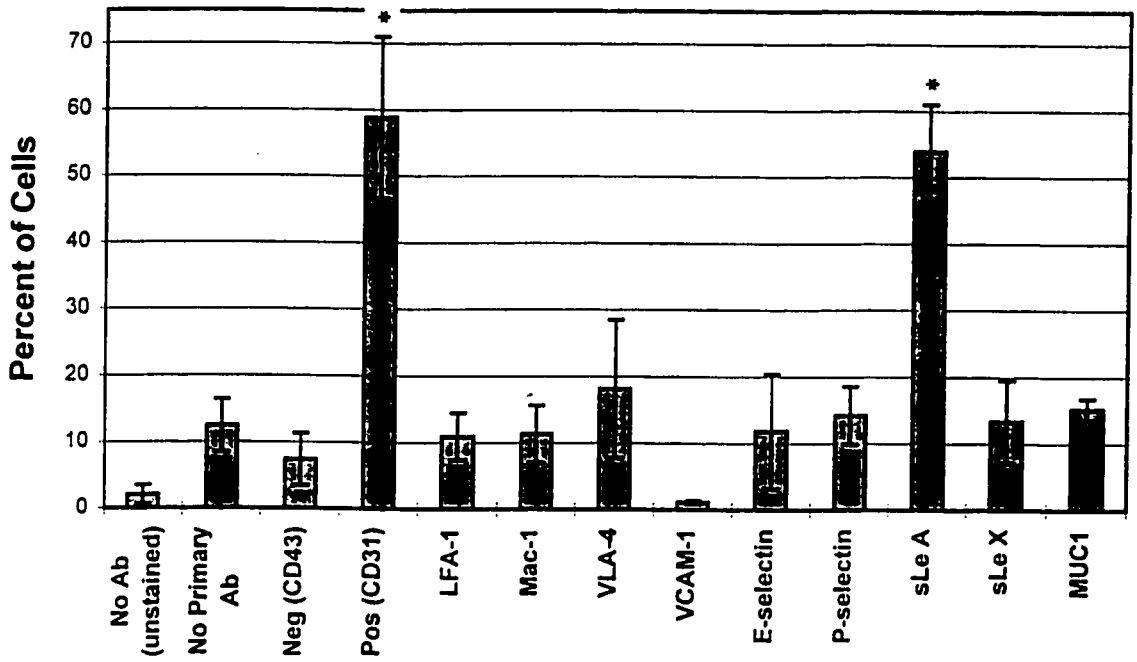


Figure 23. Surface adhesion molecule phenotype of 24h cytokine stimulated Ea-hy-926 cells analyzed by flow cytometry. "*" represents statistical significance determined by Newman-Keuls analysis ($p<0.05$). Bar graph represents the mean +/- 1 s.d. of 3 repeats.

2.3 Human Tumor Cell Adhesion to HUVECs During Physiological Shear Stress

2.3.1 Adhesion Molecule Phenotype of Human Breast Carcinoma (MCF-7)

Objective:

To phenotype the human breast carcinoma cell line, MCF-7, for MUC1 and other relevant adhesion molecules on the external cell surface.

Materials and Methods:

Cells

MCF-7 cells derived from a malignant pleural effusion of a human breast carcinoma patient (ATCC). These cells were cultured in Dulbecco's Modified Eagle's medium (DMEM)(GIBCO) supplemented with 10 % (v/v) FCS, 2mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 6 μg/ml of insulin (GIBCO).

mAbs

Monoclonal anti-MUC1 (B27.29), anti-CD43 (MT1), anti-sLe^x (CSLEX), anti-sLe^a (B67.4), anti-E-selectin (1.26B), anti-P-selectin (AK-6), anti-VLA-4 (H2/1), anti-LFA-1 (IOT-16), anti-VCAM-1 (HB9), anti-ICAM-1 (164B) and anti-MUC1 (B27.29) were used as the primary antibodies, while PE-labeled goat anti-mouse was used as the secondary antibody in the 2-step indirect FACSCan analysis method described in 2.1.1. Antibody concentrations are listed in Table 1.

Results:

The MUC1 molecule is expressed at significant levels on the MCF-7 cells in comparison to the negative control (Figure 24). Other adhesion molecules present in significant amounts on the cell surface include ICAM-1 and sLe^a.

2.3.2 Adhesion Molecule Phenotype of HUVECs

Objective:

To confirm that primary cultures of cells isolated from human umbilical veins were of endothelial origin and subsequently if these endothelial cells express increased levels of ICAM-1 or other adhesion molecules after 24 h cytokine stimulation.

Materials and Methods:

Isolation of HUVECs

Umbilical cords were stored overnight at 4^0C after delivery for a maximum of 24 h and washed in PBS prior to use. The vein of the cord was cannulated with a 21 ¾ gauge butterfly needle (Becton Dickinson) and then perfused with 20 mls of Dulbecco's PBS (DPBS)(GIBCO). The cord vein was then purged with 20 mls of air and clamped at the distal end. Approximately 3 mls of $CaCl_2$ (3mM) was infused into the vein and immediately withdrawn. A 1mg/ml solution of collagenase type I (Worthington Biochemical Corp.; Lakewood, New Jersey) was infused into the vein and the proximal end clamped. The entire cord was then submerged in DPBS and incubated for 20 min. at 37^0C. After incubation the distal end of the cord was cut and the vein (containing collagenase and harvested endothelial cells) was flushed with 20 mls DPBS. The effluent was collected in a sterile 50 ml conical tube containing 20 mls of Medium 199

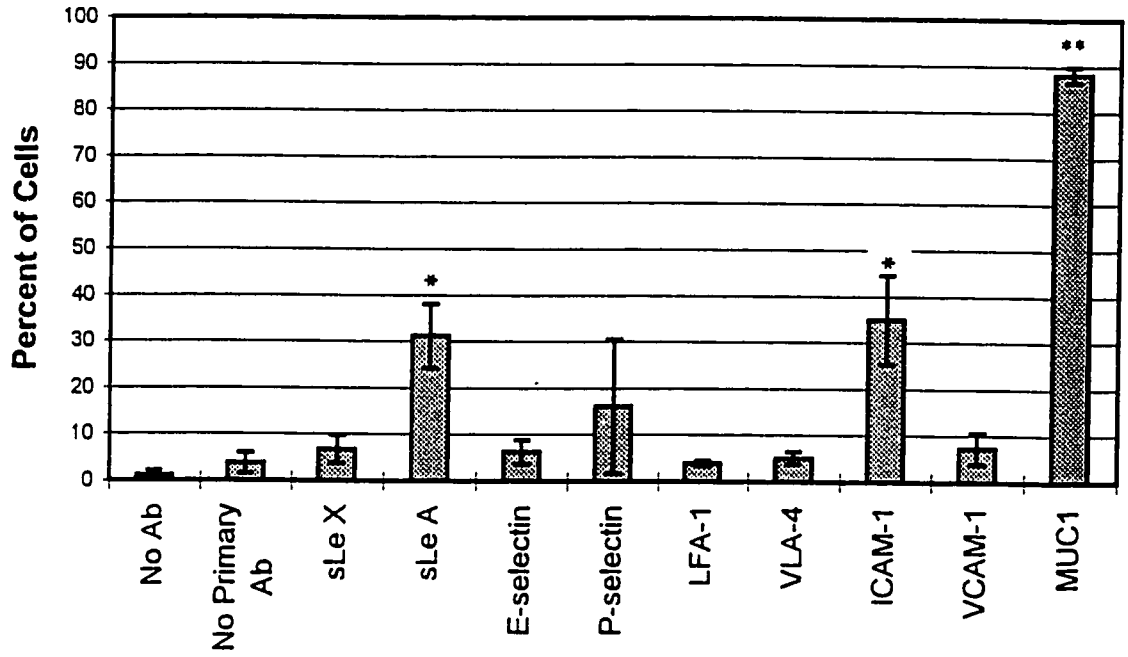


Figure 24. Surface adhesion molecule phenotype of MCF-7 tumor cells analyzed by flow cytometry. "*"-"**" represent distinct populations with statistical significance ($p<0.05$) between each as determined by Newman-Keuls analysis. Bar graph represents the mean +/- 1 s.d. of 3 repeats.

(M199)(GIBCO) supplemented with 20% (v/v) FCS, 2mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml endothelial cell growth supplement (ECGS)(Collaborative Biomedical-Becton Dickinson). The cell suspension was centrifuged at 1200 rpm for 5 min. and the supernatant was decanted. HUVECs were then cultured in supplemented M199, and analyzed for cell type by staining for endothelial cell markers or adhesion molecules using an immunoperoxidase or indirect FACScan method respectively.

Cells

HUVECs were grown in primary culture and passaged up to 3 times. Upon confluence, HUVECs were washed in Hanks Balanced Salt Solution (HBSS)(GIBCO), harvested with EDTA-trypsin, and suspended in supplemented M199. A 700 μl aliquot of HUVECs (approximately 1 x 10^6 cells/ml) was then layered onto glass slides previously coated with 0.1% (w/v) gelatin. HUVECs were then grown to confluence (4-7 days) prior to use in fluid flow adhesion assays.

Endothelial Cell Stimulation

HUVEC coated glass slides were stimulated with 700 μl 20 U/ml of both TNF-α and IL-1β for 24h at 37^{0}C prior to staining. A control, "mock" stimulation, with media and no cytokines was performed in identical conditions.

mAbs

For immunoperoxidase staining, murine monoclonal anti-LCA (2B11 and PD7/26/16), and endothelial-specific monoclonal anti-CD31 (JC/70A), anti-CD34 (8G12), anti-FVIII (F8/86) were used as primary antibodies. Horse radish peroxidase (HRP)-labeled goat anti-mouse was used as the secondary antibody in a 2-step indirect immunoperoxidase staining procedure. All antibodies listed above were the generous gift of Laith Dabbagh.

For FACScan analysis murine monoclonal anti-VCAM-1 (HB9), anti-ICAM-1 (164B), anti-sLe^x (CSLEX), anti-sLe^a (B67.4), anti-P-selectin (AK-6), anti-MUC1 (B27.29), anti-VLA-4 (H2/1), anti-LFA-1 (IOT-16), and anti-CD31 (JC/70A) were used as primary antibodies. PE-labelled goat anti-mouse was used as the secondary antibody in a 2-step indirect FACScan analysis.

Immunoperoxidase Staining (2 Step - Indirect Method)

Slides coated with isolated cord cells were dried overnight, fixed in acetone for 5 min then air dried and washed in 50 mls of PBS for 5 min. Primary murine antibodies at optimal concentrations (Table 1) were added to the slide and incubated under humid conditions at room temperature for 45 min. After washing in 50 mls of PBS for 5 min, 50 μg/ml HRP labeled goat anti-mouse secondary antibody was applied and incubated for 30 min at humid room temperature conditions. Slides were washed in PBS (5 min) and the bound labeled antibody was detected through a 5 min incubation with 3,3'-diamino-benzidine (DAB). Slides were rinsed with running tap water for 15 min, toned with 1%

(w/v) $CuSO_4$ (3 min) and rinsed again with tap water. Slides were counterstained with Harris hematoxylin (Ortho Diagnostics Systems; Ontario) for 1 min, and coverslipped after dehydration in ethanol and xylene. Molecule expression was scored according to peroxidase staining visualized by light microscopy. Slides were scored as follows: Negative (no staining), 1+ (1-10% cells/field stained), 2+ (10-40% cells/field stained), 3+ (40- 70% cells/field stained and 4+ (70-100% cells/field stained). To establish the reproducibility of the isolation protocol, cell isolates from the first 13 cords were phenotyped in triplicate.

Results:

a) Endothelial Markers

The endothelial markers CD31, CD34, and FVIII were expressed at high levels on the vast majority of cells isolated from umbilical veins (Table 2). The significant staining of these markers compared to that of the negative controls indicate that these cells are of endothelial origin and may be designated as HUVECs.

b) ICAM-1 Expression

After 24 h of cytokine stimulation, HUVECs significantly upregulated ICAM-1 expression compared to negative controls and unstimulated HUVEC monolayers (Figure 25).

c) Adhesion Molecule Phenotype

After stimulation with cytokines for 24h, HUVECs express ICAM-1, VCAM-1 and CD31 in significant amounts (Figures 25 and 26). CD31 is a homophilic adhesion molecule restricted to endothelial cells and was used as a positive control. VCAM-1 is

Staining of HUVEC cytospins						
	Antibody Added :					
Cell Type	No Ab	No Primary Ab	LCA	CD31	FVIII	CD34
HUVEC - V	NEG	NEG	NEG	4+	4+	3+
HUVEC - VI	NEG	2+	bgrd	4+	4+	n/a
HUVEC - VIII	NEG	NEG	1+	3+	4+	n/a
HUVEC - IX	NEG	NEG	1+	4+	3+	n/a
HUVEC - X	NEG	NEG	NEG	4+	4+	n/a
HUVEC - XI	NEG	NEG	NEG	4+	4+	3+
HUVEC - XII	NEG	NEG	NEG	4+	4+	3+

Table 2. Expression of endothelial cell antigens on cells isolated from human umbilical veins as analyzed by immunohistochemistry. Each preparation was made from separate a umbilical cord. Any "n/a" result had no test performed.

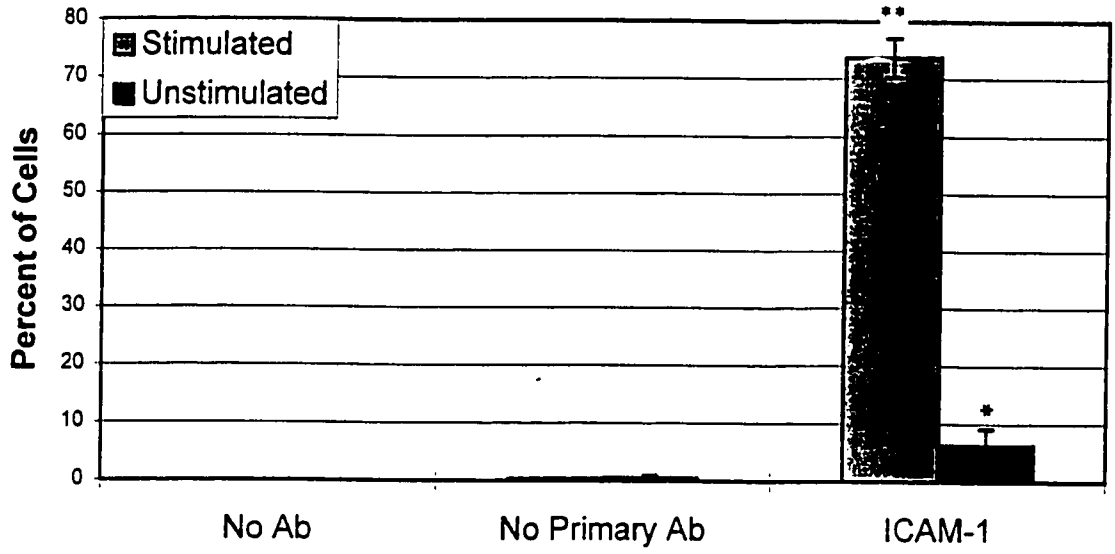


Figure 25. Surface expression of ICAM-1 on HUVECs with or without 24h cytokine stimulation as analyzed by flow cytometry. "*"-"**" represent distinct populations with statistical significance ($p<0.05$) between each as determined by Newman-Keuls analysis. Bar graph represents the mean +/- 1 s.d. of 3 repeats.

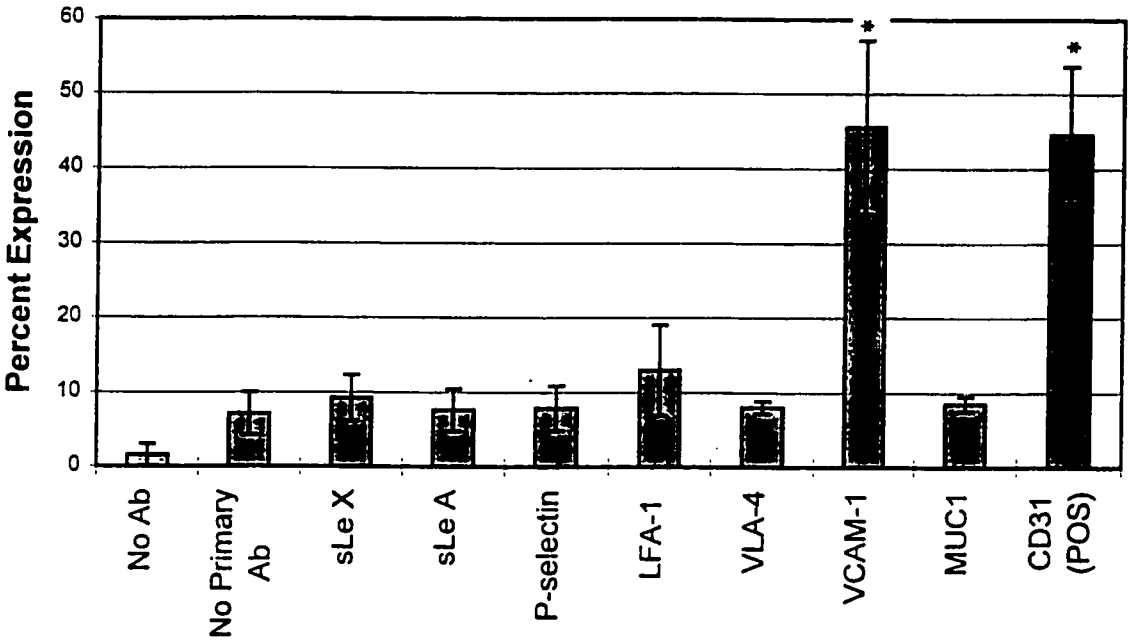


Figure 26. Surface adhesion molecule expression phenotype of 24h cytokine stimulated HUVECs monolayers. "*" represents statistical significance determined by Newman-Keuls analysis ($p<0.05$). Bar graph represents the mean +/- 1 s.d. of 3 repeats.

known to recruit immune cells to endothelium through binding of VLA-4[19,43] (which is not expressed on MCF-7 cells). Thus, adhesion ligand pairs common to inflammatory cell adhesion cascades do not exist between HUVECs and MCF-7 cells. It should be noted that E-selectin expression was not evaluated on our primary HUVEC cultures, but previous literature has proven that little or no E-selectin is expressed on HUVECs following 24 h cytokine stimulation.[84]

2.3.3 Adhesion of MCF-7 to HUVECs

Objective:

To determine if the human breast carcinoma cell line (MCF-7) can actively bind human endothelium (HUVECs) during physiological shear stress of 1 dyn/cm^2.

Materials and Methods:

Cells

MCF-7 cells were cultured as previously described. HUVECs were isolated in primary culture from umbilical veins, cultured onto glass slides and stimulated with cytokines for 24 h as previously described.

Tumor cell/endothelial cell fluid flow adhesion assay

After 24 h cytokine stimulation, HUVEC-coated glass slides were loaded onto the parallel plate flow chamber. MCF-7 cells treated with fluorescent BCECF dye were then infused into the chamber at a concentration of 0.5-1.5 x 10^5 cells/ml for 30 minutes at 37^{0}C at a shear stress of 1.0 dyn/cm^2. MCF-7 cells were put into suspension using RPMI "flow media" identical to that used for the GzHi/410.4 adhesion assays. Quantitation of

MCF-7 adherence to HUVECs was quantified by a comparison of fluorescent intensities in suspensions containing adherent tumor cells in the test to that of the control (labeled MCF-7 adhesion to stimulated HUVEC). All experiments were repeated in triplicate.

Results:

MCF-7 cells bound cytokine-stimulated endothelial monolayers 3 times greater then unstimulated endothelial monolayers (Figure 27).

2.3.4 Antibody Inhibition of Adhesion

Objective:

To determine if the recruitment of MCF-7 to cytokine stimulated HUVECs or Ea-hy-926 cells can be prevented by blockade of the MUC1 and ICAM-1 molecules.

Materials and Methods:

Cells

MCF-7 were cultured in supplemented DMEM. HUVECs harvested from primary culture in supplemented M199, were grown on 0.1% (w/v) gelatin-coated glass slides and stimulated with 20 U/ml TNF-α/IL-1β for 24 hrs. Ea-hy-926 cells were cultured, using supplemented RPMI, onto glass slides and stimulated with 20 U/ml TNF-α/IL-1β for 24 hrs.

mAbs

Either monoclonal anti-MUC1 antibody (B27.29) or a cocktail of anti-ICAM-1 antibodies (164B, 164I, 18E3D) was used in separate experiments to block MCF-

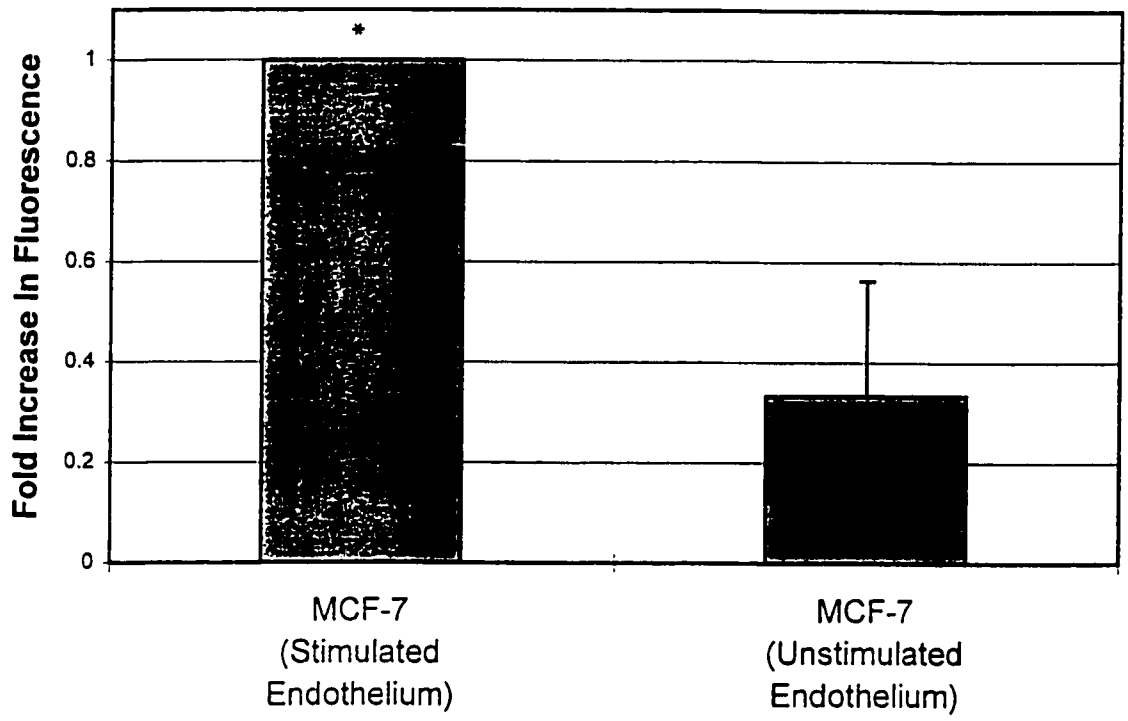


Figure 27. Adhesion of MCF-7 tumor cells to 24 h cytokine stimulated HUVECs monolayers under physiological shear stress. "*" represents statistical significance determined by Newman-Keuls analysis ($p<0.05$). Bar graph represents the mean +/- 1 s.d. of 3 repeats.

7/HUVEC or MCF-7/Ea-hy-926 adhesion as both antibody preparations were previously shown to inhibit GzHi/Ea-hy-926 adhesion.

Antibody Inhibition of Fluid Flow Adhesion Assay

MCF-7 and HUVECs were pretreated with B27.29 (anti-MUC1) antibody and a 18E3D, 164B, 164I (anti-ICAM-1) cocktail, respectively, for 30 min at 37^{0}C prior to use in parallel plate flow chamber adhesion assays.

Results:

Pretreatment of MCF-7 or HUVECs with either anti-MUC1 or anti-ICAM-1 in separate experiments did not prevent the recruitment and accumulation of MCF-7 to cytokine-stimulated HUVECs (Figure 28A). Pretreatment of cytokine-stimulated Ea-hy-926 cells with anti-ICAM-1 only minimally blocked MCF-7 adhesion (Figure 28B).

2.3.5 Summary of 2.3

MCF-7 cells are recruited in significantly greater amounts to HUVECs stimulated with cytokines for 24 hrs compared to unstimulated HUVECs. However, neither anti-MUC1 or anti-ICAM-1 pretreatment could diminish the demonstrated adhesive process. Cytokines alter the expression of numerous molecules, thus, there may be redundancy in the MCF-7/HUVEC system. As well, MCF-7 adhesion to stimulated Ea-hy-926 cells was decreased in only small amounts by anti-ICAM-1 suggesting a second adhesive process. Ligand pairs commonly involved with immune cell recruitment to endothelium are not present on these cells as demonstrated by phenotypic analysis.

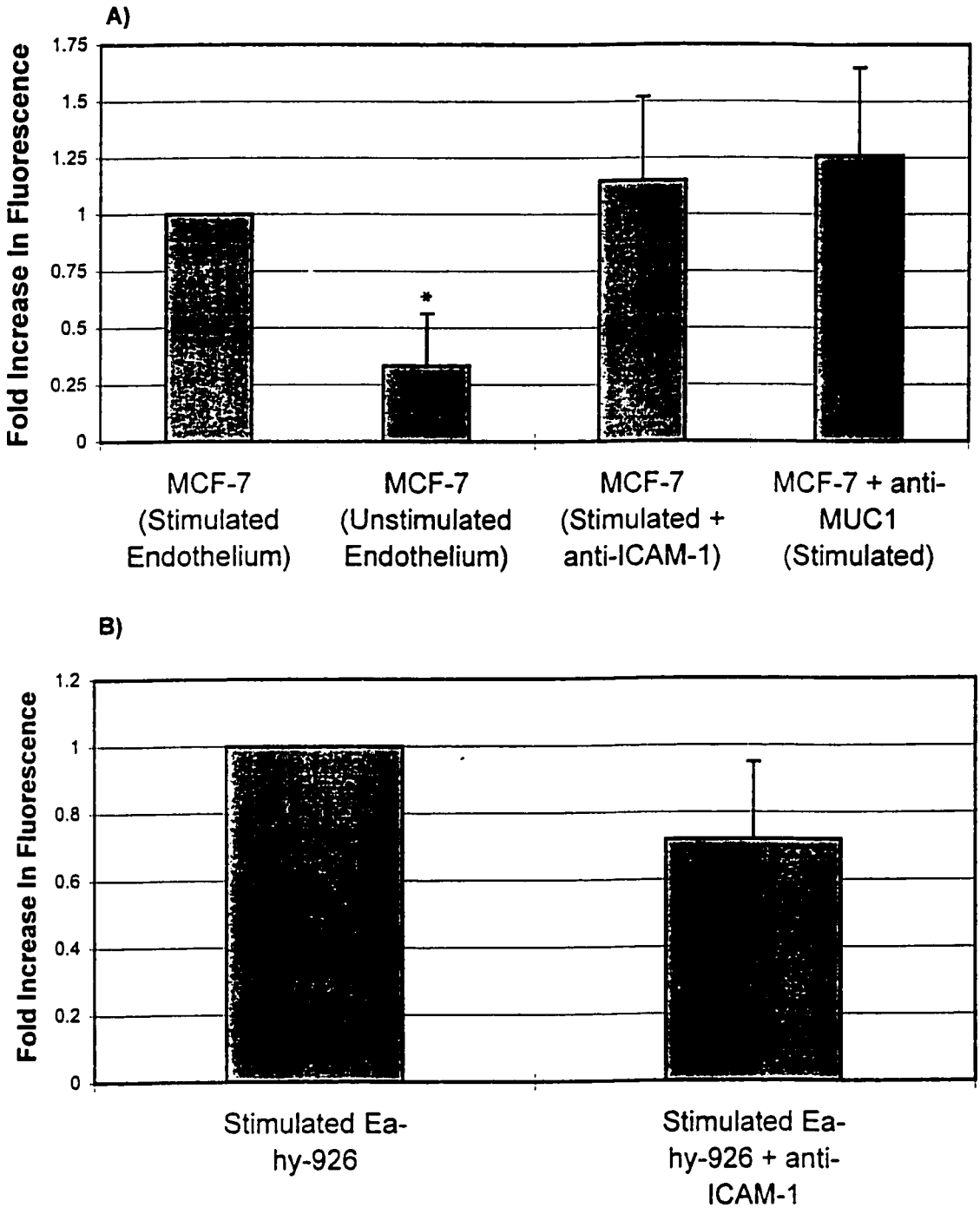


Figure 28. A) Adhesion of MCF-7 tumor cells (+/- anti-MUC1 pretreatment) to 24 h cytokine stimulated HUVECs monolayers (+/- anti-ICAM-1 pretreatment) under physiological shear stress. "*" represents statistical significance as determined by Newman-Keuls analysis. B) Adhesion of MCF-7 tumor cells to 24 h cytokine stimulated Ea-hy-926 ($p = 0.09$ via t-test). Bar graph represents the mean +/- 1 s.d. of 3 repeats.

2.4 Molecular Requirements of ICAM-1-Mediated Adhesion During Physiological Shear Stress.

2.4.1 GzHi Adhesion to Lysed Ea-hy-926

Objective:

To determine if the Ea-hy-926 recruitment of GzHi cells is dependent on an active endothelial cell process, such as intra-endothelial signaling or adhesion molecule capping with cytoskeletal rearrangement.

Materials and Methods:

Cells

GzHi and Ea-hy-926 cells were cultured in supplemented RPMI. Ea-hy-926 cells were then stimulated with 20 U/ml TNF-α/IL-1β for 24 hrs prior to lysis.

Lysed Stimulated Ea-hy-926

After 24 h cytokine stimulation, Ea-hy-926 cells were quantitated and an equal volume was a) cultured on glass slides to a confluent monolayer, or b) lysed by sonication for 1-2 min on ice. Lysed cells were transferred to microtubes and centrifuged at 10000 x g for 10 minutes. After decanting the supernatant, crude Ea-hy-926 membranes were resuspended in PBS, coated onto polystyrene (plastic) slides (Nagle Nunc International; Naperville, Illinois), dried overnight and used in adhesion assays after resolubilization with HBSS containing 1.2 mM Ca^{2+} and 1.0 mM Mg^{2+}.

Results:

GzHi cells bind to both live and lysed stimulated Ea-hy-926 monolayers without significant difference (Figure 29). ICAM-1 presence on lysed endothelial membranes was confirmed by immunostaining (data not shown). Thus, adhesion of GzHi cells is not dependent on an active process within live endothelial cells.

2.4.2 GzHi Adhesion to Immobilized rhICAM-1

Objective:

To determine if GzHi adhesion to Ea-hy-926 in physiological fluid flow is mediated solely by the ICAM-1 molecule on endothelium.

Materials and Methods:

Cells

GzHi cells were cultured as previously described.

mAbs

ELISA assays employed a primary monoclonal anti-ICAM-1 (18E3D) and HRP conjugated goat anti-mouse as a secondary (gift of Dr. C. Cass: Cross Cancer Institute; Edmonton, Alberta). Flow cytometry used the 18E3D antibody along with the anti-MUC1 antibody B27.29. The 18E3D antibody was chosen for this experiment as it has previously been shown to be specific for the ICAM-1 epitope responsible for MUC1 binding.[84] PE labeled goat anti-mouse was used as the secondary antibody.

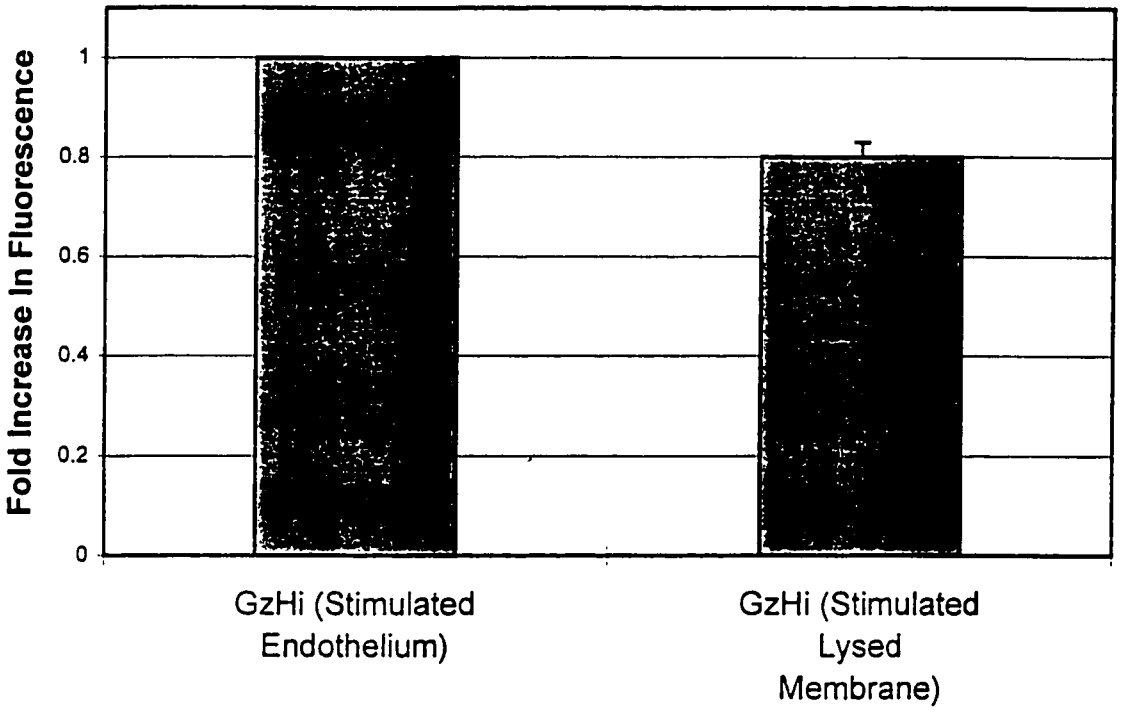


Figure 29. Adhesion of GzHi tumor cells to stimulated Ea-hy-926 cells, live and lysed, under physiological shear stress. No statistical significance, as determined by Newman-Keuls analysis ($p>0.05$). Bar graph represents the mean +/- 1 s.d. of 3 repeats.

Reagents

rhICAM-1 (gift of ICOS Corp.) was used in the adhesion assays as well as in the quantitative ELISA procedures. The rhICAM-1 molecule consists of two ICAM-1 molecules fused to the Fc portion of a human immunoglobulin. Collagen type I (Celltrix Vitrogen 100) was used as a control protein in the fluid flow adhesion assays.

Coating rhICAM-1 and Collagen to Plastic Slides

Polystyrene slides were coated with 500 µl of 20 µg/ml rhICAM-1 or 500 µl of 10 µg/ml collagen type I and dried overnight at room temperature. Prior to adhesion assays the slide was resolubilized in HBSS containing 1.2 mM Ca^{2+} and 1.0 mM Mg^{2+} and blocked with 0.1% (w/v) BSA in PBS for 30 min at 37°C prior to use in fluid flow adhesion assays.

Enzyme Linked Immunosorbent Assay (ELISA)

96 well microtitre plates (Corning Costar; Acton, Massachusetts) were coated with 100 µl of 1.0 µg/ml of rhICAM-1 and incubated at 4°C overnight. After washing (x2) in PBS, blocking in 5% BSA (w/v) for 1 h at 37°C, and further PBS washing (x2), primary antibody (18E3D) was added at varying dilutions (0.2-1.0 µg/ml) and incubated at 4°C overnight. After washing in PBS (x5), secondary GAM antibody labeled with HRP was added at varying dilutions (1/5000-1/20000) and incubated for 1 h at 37°C. ELISA plates were then washed (x5) in PBS and color was developed by adding 100 µl from a fresh solution containing 1 mg 3,3',5,5'-tetramethylbenzidine dihydrochloride

(TMB)(Sigma), 2 μl of 30% (v/v) hydrogen peroxide and 10 ml phosphate citrate buffer at pH = 5.0. After developing in the dark for 15 min the reaction was halted by addition of 25 μl of 2M H_2SO_4 for 5 min. The optical density of color development was measured using a plate reader (laboratory of Dr. L.M. Pilarski) at 450 ηm. All ELISA assays were repeated in triplicate.

Results:

a) rhICAM-1 Immunoreactivity and Presence on Polystyrene Plates

The optical density increased linearly with decreasing dilutions of the secondary antibody (Figure 30). In the absence of any primary antibody against ICAM-1 only background optical density levels were present. These ELISA results indicate that the rhICAM-1 is immunoreactive and likely conformationally intact. This also demonstrates that the coating procedure for the fluid flow adhesion assays results in significant deposition of immobilized rhICAM-1 on plastic slides.

b) Fluid Flow Adhesion Assay

There was no difference between rhICAM-1 and collagen in their ability to recruit GzHi cells (Figure 31) indicating that GzHi adherence is not enhanced by the rhICAM-1 coated slides.

c) Pre/Post Flow Adhesion Assay Analysis of GzHi

FACScan analysis of GzHi cells pre- and post-fluid flow adhesion assay on collagen and rhICAM-1 slides showed no difference in expression of ICAM-1 and an increase in MUC1 expression (Figure 32). This eliminates the possibility that the shear stress led to detachment of ICAM-1 preventing the retention of bound GzHi cells on

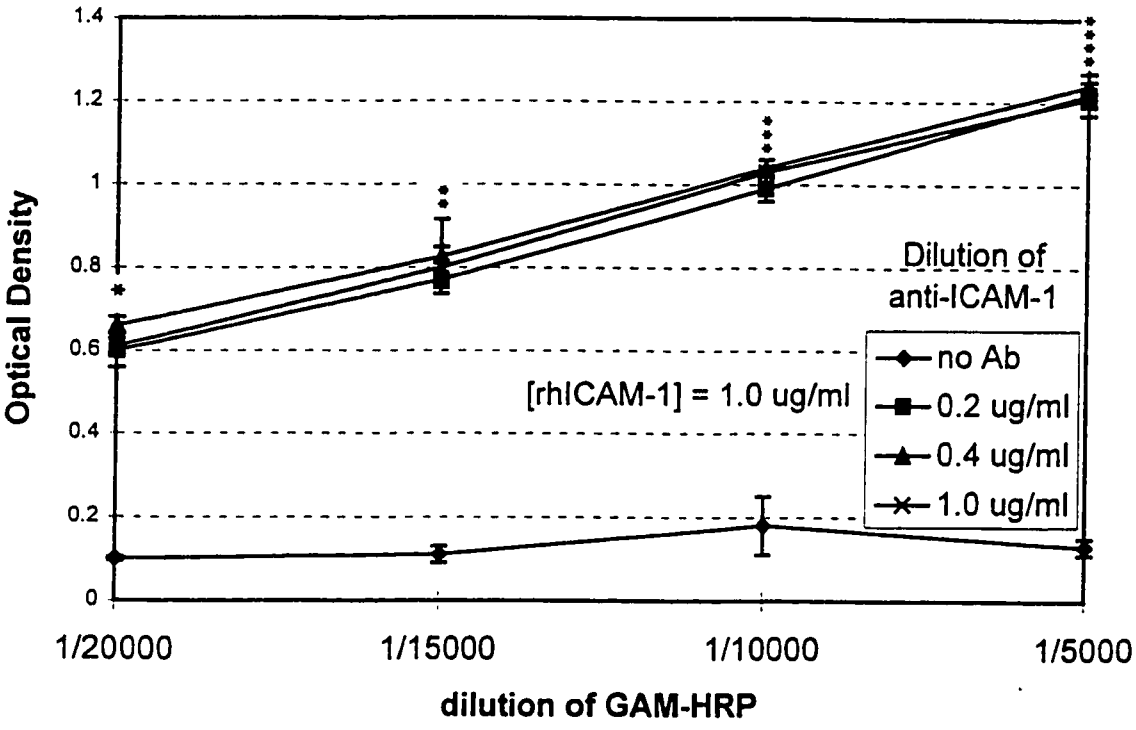


Figure 30. Immunoreactivity of the rhICAM-1 molecule as determined by ELISA. "*"-"****" represent distinct populations with statistical significance ($p<0.05$) between each as determined by Newman-Keuls analysis. Graph represents the mean +/- 1 s.d. of 3 repeats.

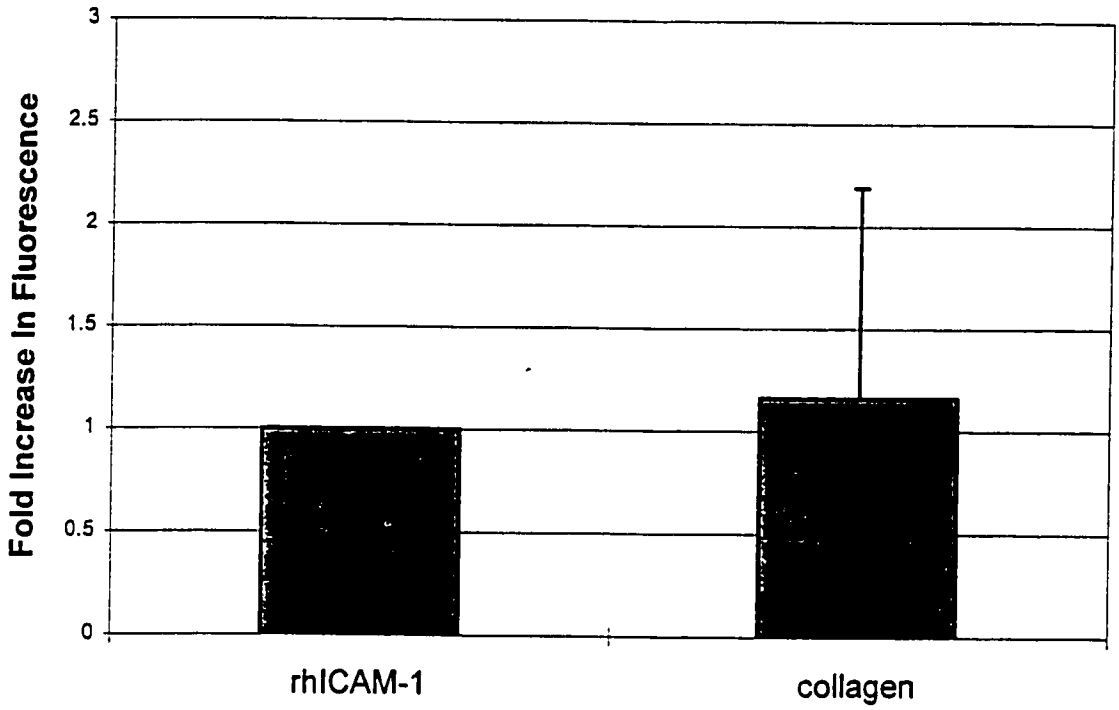


Figure 31. Recruitment of GzHi tumor cells to immobilized rhICAM-1 and collagen coated slides under physiological shear stress. No statistical significance as determined by t-test ($p>0.05$). Bar graph represents the mean +/- 1 s.d. of 3 repeats.

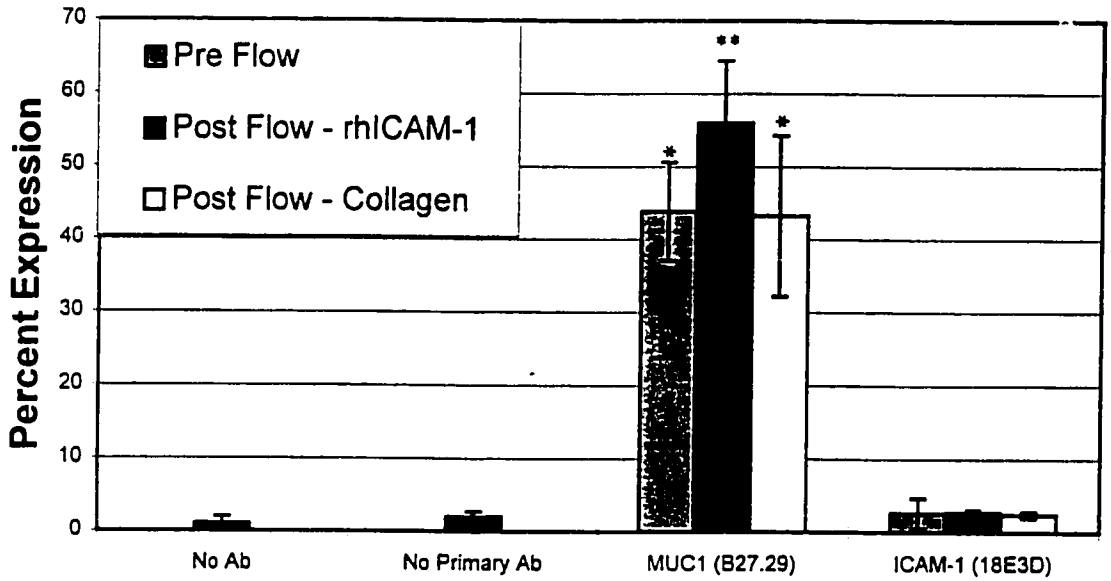


Figure 32. Presence of MUC1 and ICAM-1 on GzHi tumor cells pre/post fluid flow over immobilized rhICAM-1 or collagen coated slides. "*"-"**" represent distinct populations with statistical significance ($p<0.05$) between each as determined by Newman-Keuls analysis. Bar graph represents the mean +/- 1 s.d. of 3 repeats.

rhICAM-1 coated plastic slides in fluid flow (Figure 33).

2.4.3 GzHi Adhesion to High Density Immobilized rhICAM-1

Objective:

To determine if rhICAM-1 coated at increased density, similar to that on stimulated Ea-hy-926 cells, recruit GzHi tumor cells with similar efficiency. Adhesion surfaces were coated with GAH antibody which served to bind the Ig portion of the rhICAM-1 molecule, properly orientating the ICAM-1 segment of the molecule for maximum adhesion and increasing the ICAM-1 density as two rhICAM-1 molecules will bind to every GAH (Figure 34).

Materials and Methods:

Cells

GzHi and Ea-hy-926 cells were cultured in supplemented RPMI. Ea-hy-926 cells were stimulated with TNF-α/IL-1β for 24 hrs..

mAbs

Anti-ICAM-1 (18E3D) and HRP-conjugated goat anti-mouse antibodies were used in ELISA assays.

Reagents

The ELISA assay requires rhICAM-1 (ICOS Corp.), 5% (w/v) BSA in addition to goat anti-human (GAH)(Sigma) antibody specific for the Fc portion of human Ig..

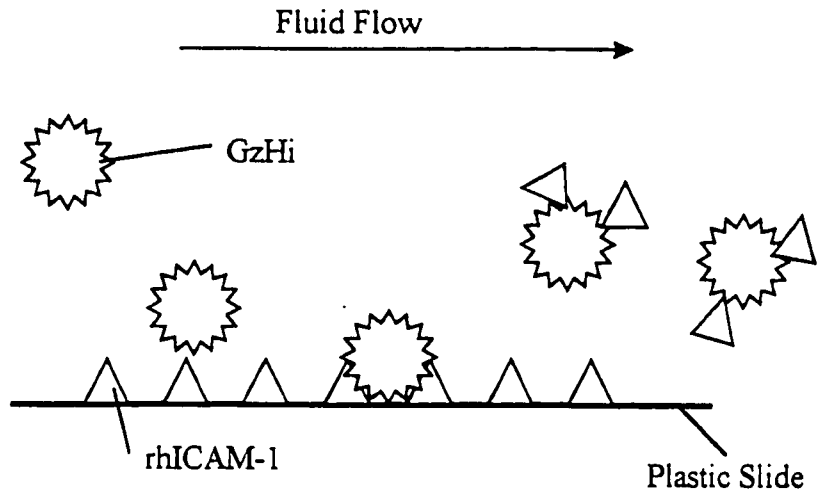


Figure 33. Theoretical mechanism of rhICAM-1 binding MUC1 on GzHi tumor cells without causing adhesion of cells to plastic slides during physiological shear stress.

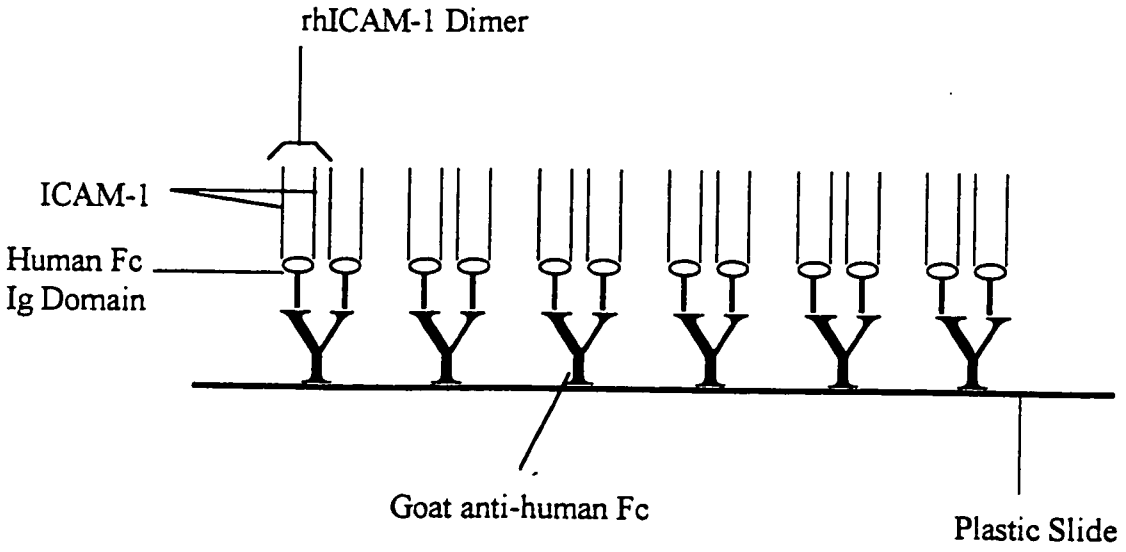


Figure 34. Mechanism of increased ICAM-1 density on slides pretreated with GAH specific for the Fc portion of the rhICAM-1 molecule.

ELISA

In the first ELISA procedure microtitre plates were coated at 4°C overnight with 100 µl of GAH antibody (0.5-7.0 µg/ml) specific for the Fc portion of human Ig. Plates were then washed (x2) in PBS and blocked in 5% (w/v) BSA for 1 hr at 37°C. After washing (x2) in PBS, 3.5 µg/ml of rhICAM-1 was added overnight at 4°C to bind the GAH molecule. The deposited rhICAM-1 was detected using 4 µg/ml of anti-ICAM-1 (18E3D) and 1/5000 GAM-HRP which produces a color reaction with the TMB substrate. The positive control (no GAH) wells were coated with only 3.5 µg/ml of rhICAM-1 prior to antibody addition.

In a parallel ELISA assay Ea-hy-926 cells were grown to confluence in culture, stimulated for 24 hrs with 20 U/ml TNF-α /IL-1β and lysed by sonication. Membrane fractions were then plated in ELISA wells. The volume of cells added per area of plastic well was the same used for coating lysed cells to plastic slides. In parallel wells, GAH/rhICAM-1 was coated at a 1:2 molecular ratio using 100 µl of 2.0 µg/ml GAH and 4.0 µg/ml rhICAM-1, determined to be optimal by the preceding ELISA results. Anti-ICAM-1 (18E3D) was used at varying dilutions (2-10 µg/ml) and 1/5000 GAM-HRP was used as the secondary antibody. 5% (w/v) BSA was used to block GAH-and Ea-hy-926-coated wells, while all PBS washes were performed in an identical manner to the preceding ELISA assay. ICAM-1 density of each was then determined by the optical density of the color reaction produced by the HRP substrate TMB.

Coating Plastic Slides

Polystyrene slides were prepared with a protocol similar to that used for the ELISA assay. 10 μg/ml of Ig-specific GAH was coated on the plastic slide and incubated at 4°C overnight. The slide was then blocked with 2 % (w/v) BSA for 1 hr at 37°C and then incubated with 20 μg/ml of rhICAM-1 (using a 2:1 ratio of rhICAM-1:GAH) at 4°C overnight. The following day these high density rhICAM-1 slides were used in fluid flow adhesion assays.

Results:

a) ICAM-1 Density Comparison of GAH/rhICAM-1 to rhICAM-1 Coated Wells.

An rhICAM-1:GAH ratio of 2:1 was optimal for maximizing ICAM-1 density. Optical density of the "high density" ICAM-1 (GAH/rhICAM-1) coated wells at this predetermined ratio was increased compared to wells coated with rhCAM-1 alone (no GAH)(Figure 35).

b) ICAM-1 Density Comparison of GAH/rhICAM-1 to Stimulated Ea-hy-926 cells Coated Wells.

Optical density of the "high density" (GAH/rhICAM-1) ICAM-1 plates was significantly increased compared to cytokine stimulated Ea-hy-926 coated wells (Figure 36). Thus, the high density slides used in the fluid flow adhesion assays then reflect physiological or supra-physiological levels of ICAM-1 density.

c) GzHi adhesion to GAH/rhICAM-1 vs. GAH coated slides

Under physiological shear stress conditions, the high density ICAM-1 slides did not bind GzHi cells in greater amounts than slides coated with GAH (Figure 37).

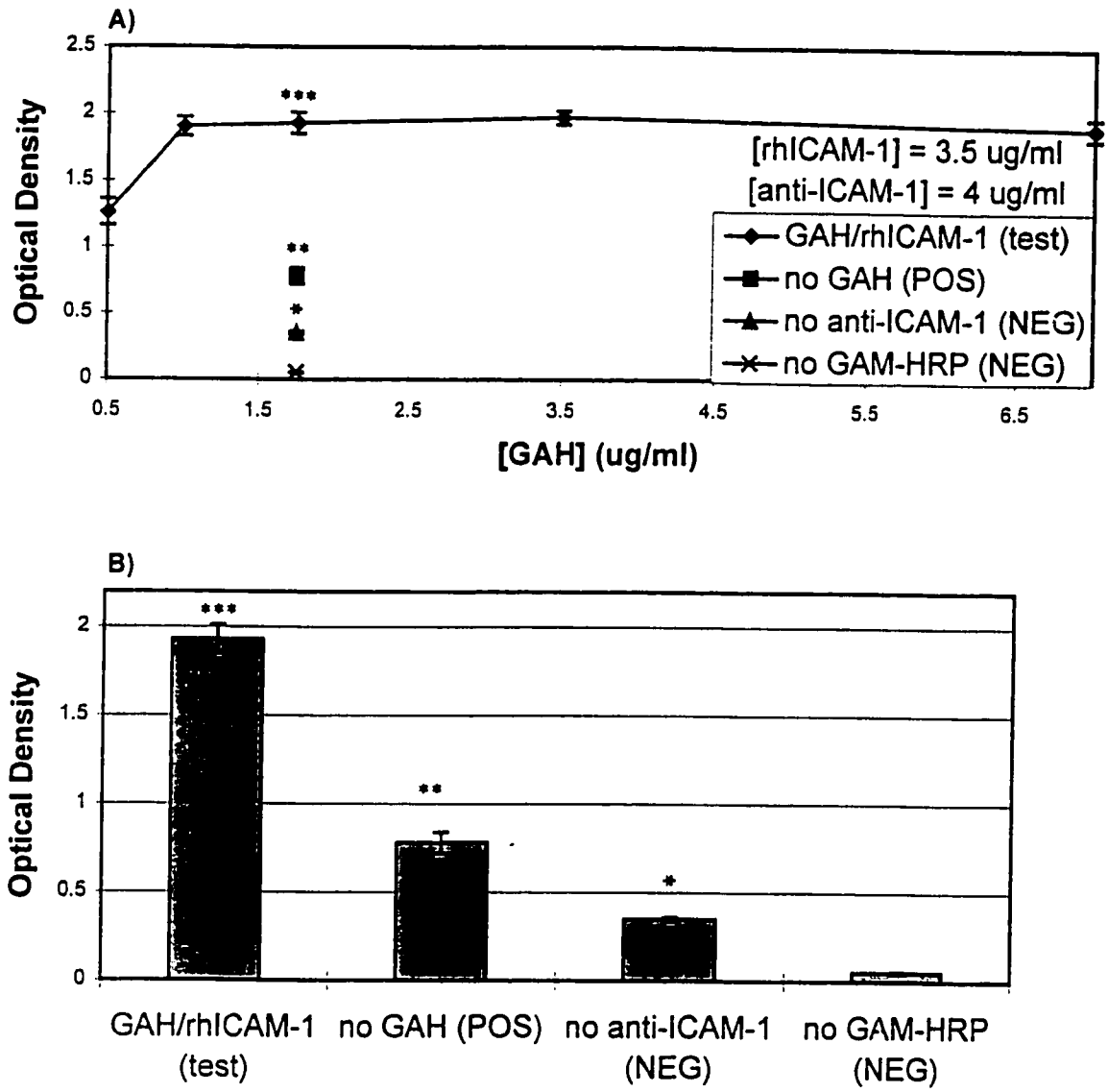


Figure 35. ICAM-1 density of GAH/rhICAM-1 coated slides compared to slides coated with rhICAM-1 alone as determined by ELISA. A) Plot of GAH coated in varying dilution has maximum binding of 3.5 μg/ml rhICAM-1 at 1.75 μg/ml of GAH. Negative control samples and a positive control were performed at this concentration as well. B) Bar graph of the optical densities corresponding to wells coated with 1.75 μg/ml of GAH and subsequently coated with other constituents of the ELISA assay, except where indicated. "*"-"***" represent distinct populations with statistical significance ($p<0.05$) between each as determined by Newman-Keuls analysis. Graphs represent the mean +/- 1 s.d. of 3 repeats.

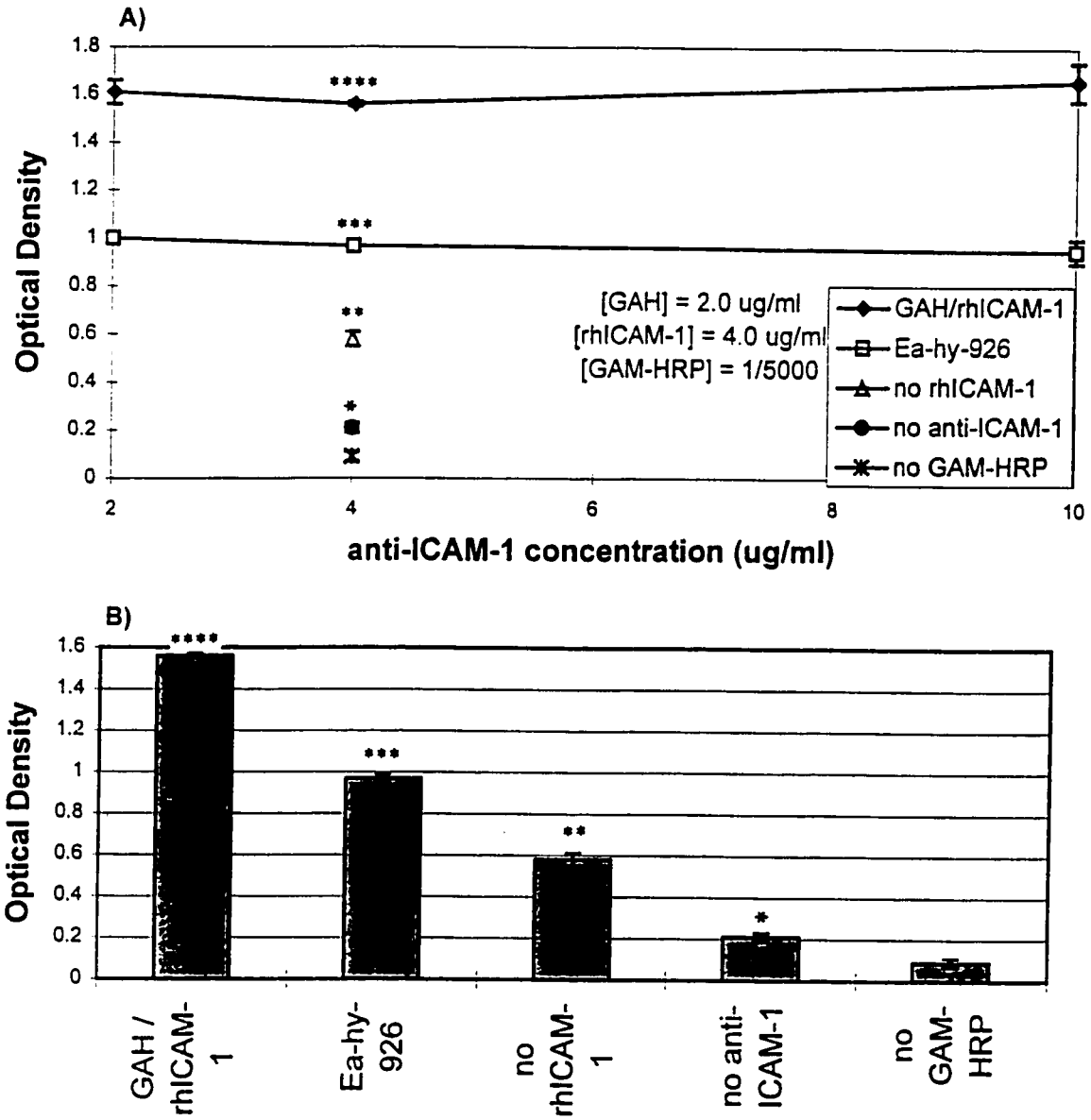


Figure 36. ICAM-1 density of GAH/rhICAM-1 coated slides compared to slides coated with lysed 24h cytokine stimulated Ea-hy-926 cells, as determined by ELISA. A) Plot of ICAM-1 density comparisons using constant levels of all ELISA reagents except anti-ICAM-1. B) Bar graph of ICAM-1 optical densities corresponding to 4 μg/ml of anti-ICAM-1. All other constituents were present (at constant levels) except where indicated. "*"-"****" represent distinct populations with statistical significance ($p<0.05$) between each as determined by Newman-Keuls analysis. Graphs represent the mean +/- 1 s.d. of 3 repeats.

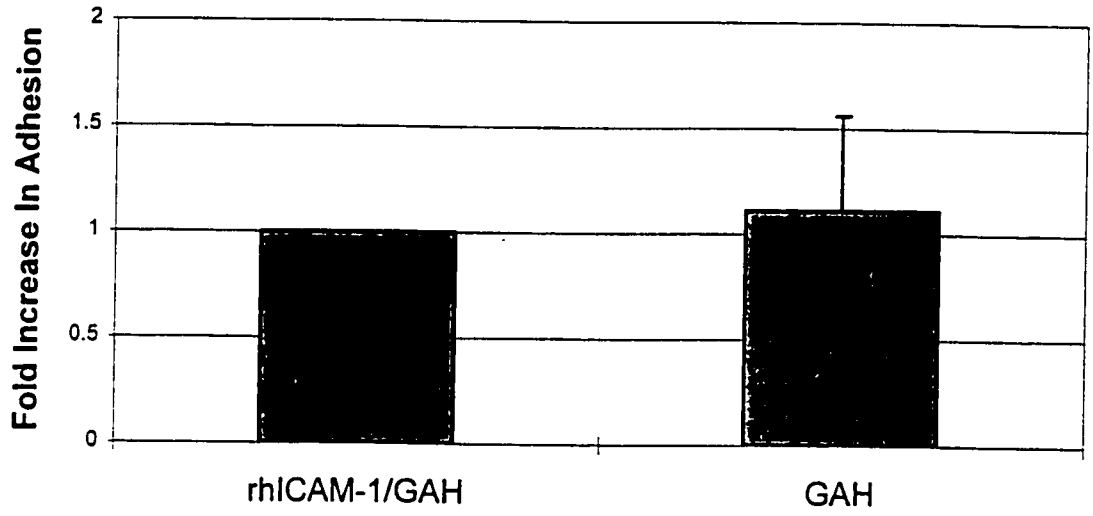


Figure 37. Recruitment of GzHi tumor cells to high density ICAM-1 (GAH/rhICAM-1) slides compared to slides coated with GAH alone under physiological shear stress. No statistical significance, as determined by t-test ($p>0.05$). Bar graph represents the mean +/- 1 s.d. of 3 repeats.

2.4.4 Summary of 2.4

The adhesion of GzHi cells can occur on lysed membranes prepared from stimulated endothelial cells, thus no active process within live endothelial cells is required for ICAM-1 mediated binding, However, plates coated with purified rhICAM-1 at densities similar to that of the endothelial cell membranes could not bind the MUC1 expressing GzHi cells. Furthermore, the inability of rhICAM-1 to bind GzHi cells was not due to weak rhICAM-1 immobilization onto plastic slides. No increase in ICAM-1 presence on GzHi cells was observed after fluid flow adhesion assay. This eliminates the detachment of bound rhICAM-1 from the plastic slide due to shear force upon GzHi adherence as a reason for the lack of adhesion. Thus, we were unable to demonstrate that ICAM-1-mediated adhesion of tumor cells under physiological shear stress could occur with the purified ICAM-1 protein alone.

2.5 Molecular Requirements of MUC1-Mediated Adhesion During Physiological Shear Stress

2.5.1 GzHi Adhesion to Cytokine Stimulated Ea-hy-926 After sMUC1 Pretreatment

Objective:

To inhibit the adhesion of GzHi tumor cells to stimulated Ea-hy-926 monolayers by occupying the ICAM-1 ligand on endothelial cells with sMUC1.

Materials and Methods:

Cells:

GzHi and Ea-hy-926 cells were cultured in supplemented RPMI. Ea-hy-926 cells

were stimulated with 20 U/ml TNF-α/IL-1β for 24 hrs.

sMUC1 Pretreatment

After 24 h of cytokine stimulation, Ea-hy-926 monolayers were treated with 200 U/ml sMUC1 (gift of Biomira, Inc.) for 30 min at 37^{0}C prior to use in fluid flow adhesion assays.

Results:

Pretreatment with sMUC1 had no effect on the adhesion of GzHi cells to stimulated monolayers (Figure 38). Immunostaining monolayers for the presence of MUC1 before and after fluid flow determined that while in static conditions sMUC1 did bind Ea-hy-926, after 30 min of fluid flow no sMUC1 remained on the endothelium and thus could not inhibit GzHi adhesion.

2.5.2 GzHi Adhesion to Cytokine Stimulated Ea-hy-926 After Milk Fat Globule Pretreatment

Objective:

To determine if the incorporation of MUC1 into a lipid bi-layer allows for high affinity MUC1/ICAM-1 binding and blockade of GzHi adhesion with pretreatment of MFG.

Materials and Methods:

Cells

GzHi and Ea-hy-926 cells were cultured in supplemented RPMI. Ea-hy-926 cells

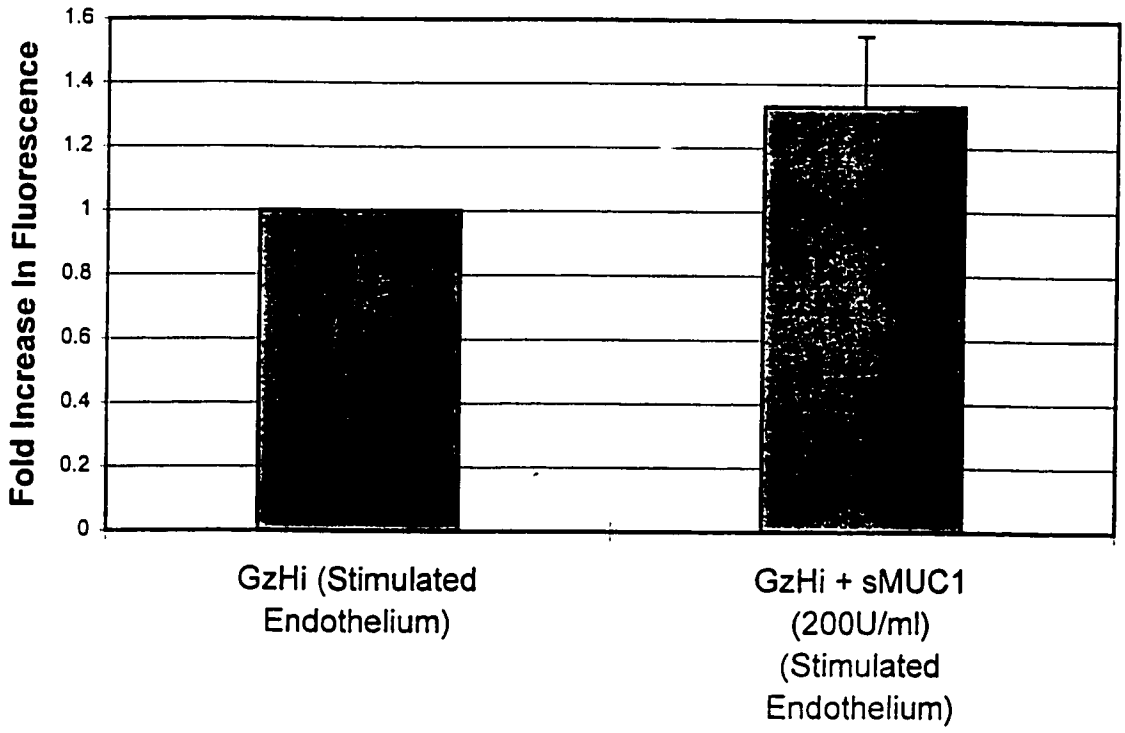


Figure 38. GzHi tumor cell adhesion to Ea-hy-926 monolayers stimulated with cytokines for 24h (+/- pretreatment with sMUC1) under physiological shear stress. No statistical significance as determined by Newman-Keuls analysis ($p>0.05$). Bar graph represents the mean +/- 1 s.d. of 3 repeats.

were stimulated with 20 U/ml TNF-α/IL-1β for 24 hrs.

Milk Fat Globule Pretreatment

Other members of this laboratory isolated milk fat globule (MFG) by ultracentrifugation of breast milk from normal human females. This MFG preparation was used as a crude source of liposomal MUC1. After 24 h cytokine stimulation Ea-hy-926 monolayers were treated for 30 min at 37°C with 500 µl of MFG diluted 1:2 in PBS. Monolayers were washed in PBS (x2) prior to use in fluid flow adhesion assays.

Protein Electrophoresis and Immunoblot

MFG preparations and purified sMUC1 were homogenized and separated by SDS-PAGE.[100] Briefly, wells of the 6% (w/v) acrylamide gel were loaded individually with either molecular weight standards, MFG or sMUC1. After separation, gels were either stained or transferred to nitrocellulose for immunoblot analysis.

Silver Stain of 6% (w/v) Acrylamide Gel

Protein bands were visualized using a standard silver stain protocol. Gels were fixed in solution containing 50% (v/v) ethanol, 10% (v/v) acetic acid and 40% (v/v) H_2O. After decanting and rinsing in 30% (v/v) ethanol gels were washed (x3) in distilled water (DH_2O) prior to sensitization with 0.2 g/L sodium thiosulphate. Gels were again washed in DH_2O before staining with 2.0 g/L silver nitrate. After a final DH_2O wash, color was developed on the stained protein by incubation with a developing solution (60g/L

Na_2CO_3, 20 ml/L sensitizing solution, and 500 µl/L 37% (w/v) formaldehyde). Color development was stopped using 6% (v/v) acetic acid. Gels were then washed in DH_2O and dried under cellophane.

Nitrocellulose Immunoblot

Blots were stained for MUC1 using a multi-step alkaline phosphatase immunoblot procedure. After incubation in blocking buffer (5% (w/v) non-fat dry milk, 0.05% (v/v) Tween 20 in PBS) primary B27.29 (anti-MUC1) diluted in blocking buffer was added for 30 minutes at 37^{0}C and then washed with blocking buffer. Biotin labeled GAM (1/10000; 10 mls/test)(Jackson Immuno Research; West Grove, Pennsylvania) was then incubated with the blot and subsequently washed with blocking buffer, followed by washing with Tris Buffered Saline (TBS). Streptavidin - Alkaline Phosphatase (Boehringer Mannhein; Germany) conjugate was then incubated with the blot and subsequently washed in TBS after incubation. Finally the color was developed by incubating the alkaline phosphatase substrate and color reactants 2% NBT/BCIP (nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt) staining solution until desired color development. Blots were then washed in H_2O and dried.

Results:

a) Adhesion after MFG pretreatment

GzHi adhesion to stimulated Ea-hy-926 during physiological shear stress was not inhibited by pretreatment of endothelium with MFG prior to fluid flow(Figure 39).

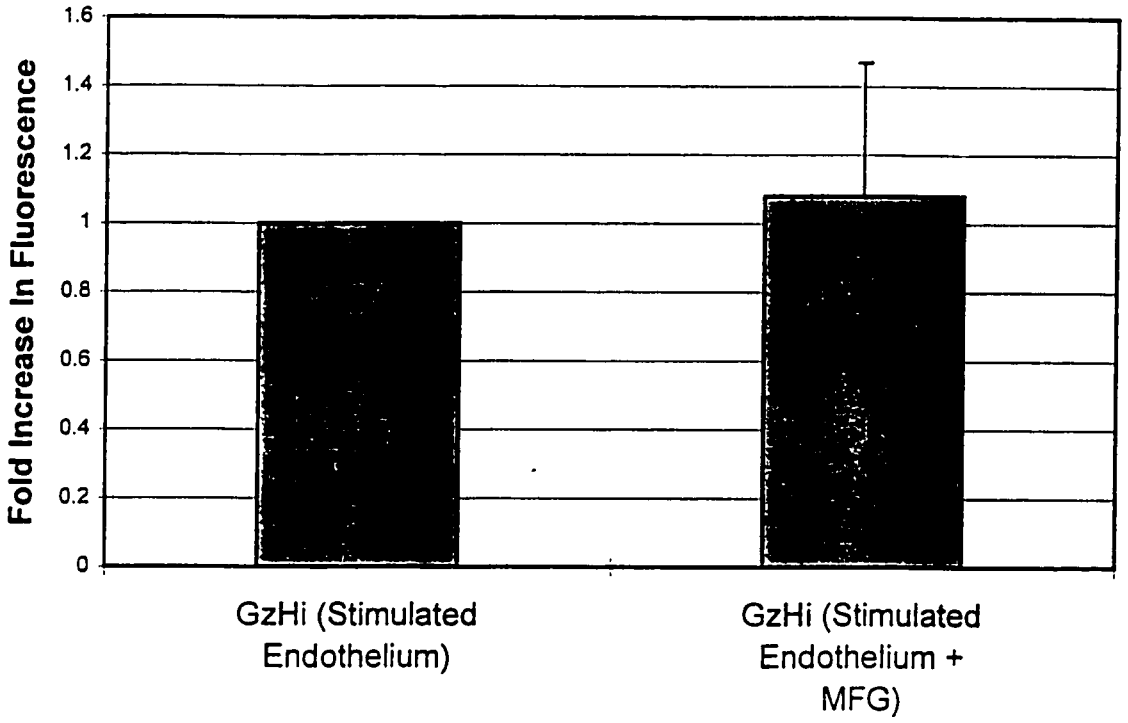


Figure 39. GzHi tumor cell adhesion to 24 h cytokine stimulated Ea-hy-926 monolayers (+/- pretreatment with MFG) under physiological shear stress. No statistical significance as determined by Newman-Keuls analysis ($p>0.05$). Bar graph represents the mean +/- 1 s.d. of 3 repeats.

b) MUC1 presence in MFG preparations

SDS-PAGE and subsequent immunoblot analysis of MFG and sMUC1 with anti-MUC1 determined that MUC1 is present in MFG and sMUC1 preparations (Figure 40). Using molecular weight standards, the approximate size of the MFG MUC1 molecule is 310 kD while the sMUC1 molecule used previously was 300 kD , which is within the accepted range for the human MUC1 molecule.

2.5.3 Summary of 2.5

Pretreatment of 24 hour cytokine-stimulated endothelium with either sMUC1 or MFG could not prevent the recruitment of GzHi cells during fluid flow. SDS-PAGE and immunoblot analysis confirmed that MUC1 was present in the MFG and sMUC1 preparations. Staining the monolayers pre and post fluid flow after treatment with sMUC1 revealed that MUC1 was present on the cells only prior to fluid flow (data not shown). This suggests that binding of each preparation was transient and washed off during continuous fluid flow.

2.6 Effect of Shear Stress on GzHi Adhesion to Stimulated Ea-hy-926.

Objective:

To determine if MUC1/ICAM-1 adhesion occurs at shear stresses similar to that of leukocytes utilizing other adhesion molecules.

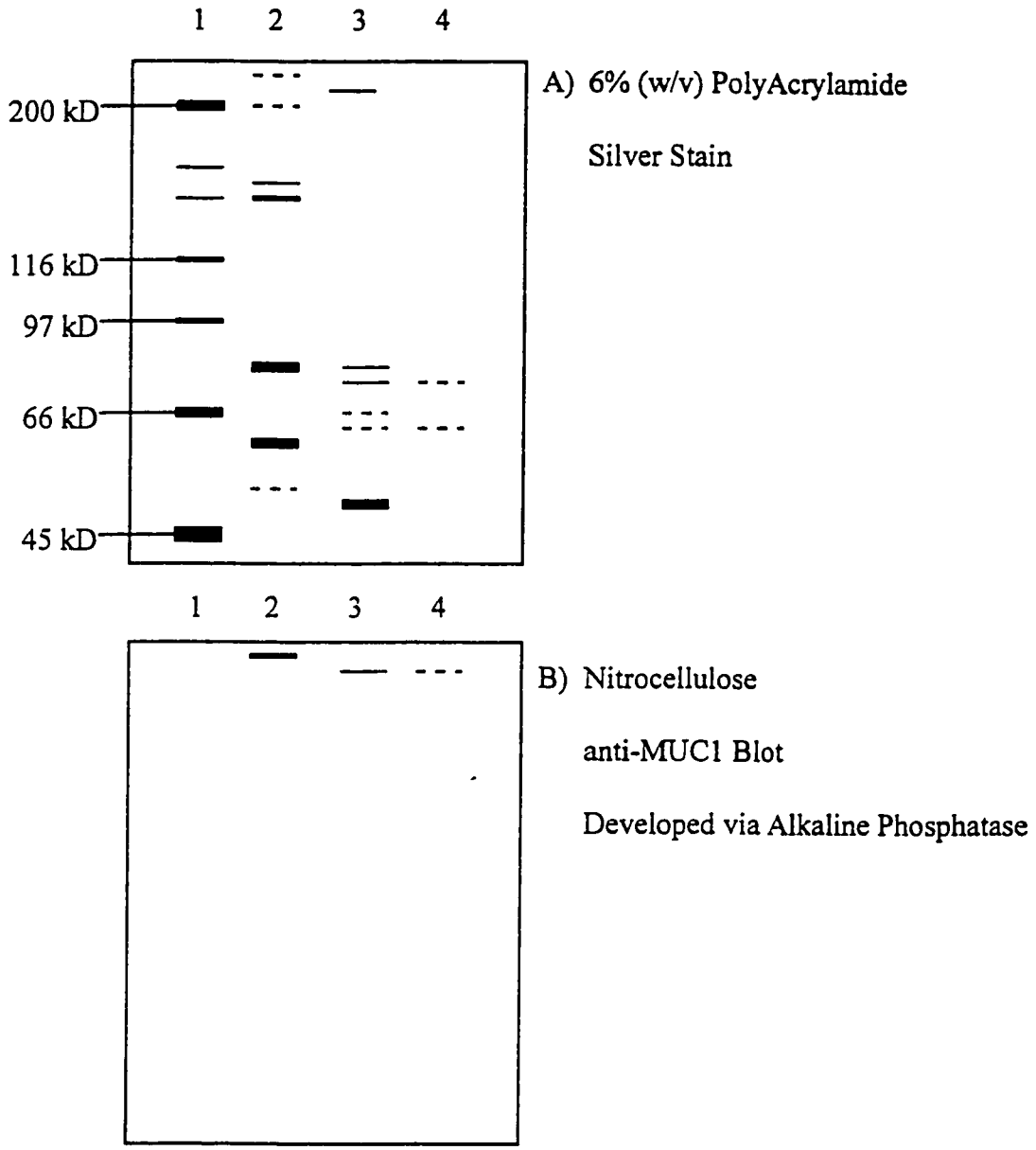


Figure 40. A) SDS-PAGE and silver stain analysis of proteins present in samples: 1) M.W. standards, 2) MFG, 3) sMUC1 (PE311) and 4) sMUC1 (PE340). B) Nitrocellulose transfer and immunoblot using anti-MUC1 (B27.29) on sample lanes identical to those of the above gel. Note: the immunoblot procedure appears more sensitive as lane 4 has a MUC1 band not present with silver stain.

Materials and Methods:

Cells

GzHi and Ea-hy-926 cells were cultured in supplemented RPMI. Ea-hy-926 cells were stimulated with 20 U/ml TNF-α/IL-1β for 24 hrs.

Fluid Flow Adhesion Assay

Adhesion assays were performed as previously described with volumetric flow rate (which determines shear stress) as the only variable between experiments. Shear stresses of 0.5 dyn/cm^2 (sub-physiological), 1.0 dyn/cm^2, 3.0 dyn/cm^2 and 5.0 dyn/cm^2 (physiological)[21] were used in the experiments.

Results:

Shear stress is a determining factor in the recruitment of GzHi tumor cells to 24h stimulated Ea-hy-926 monolayers (Figure 41). Sub-physiological (0.5 dyn/cm^2) shear stress allowed for 2 fold more adhesion of GzHi cells compared to physiological levels of 1.0 dyn/cm^2 and 3.0 dyn/cm^2. At shear stress levels of 5.0 dyn/cm^2 the adhesion of GzHi tumor cells was significantly reduced compared to that of lower physiological shear stress levels.

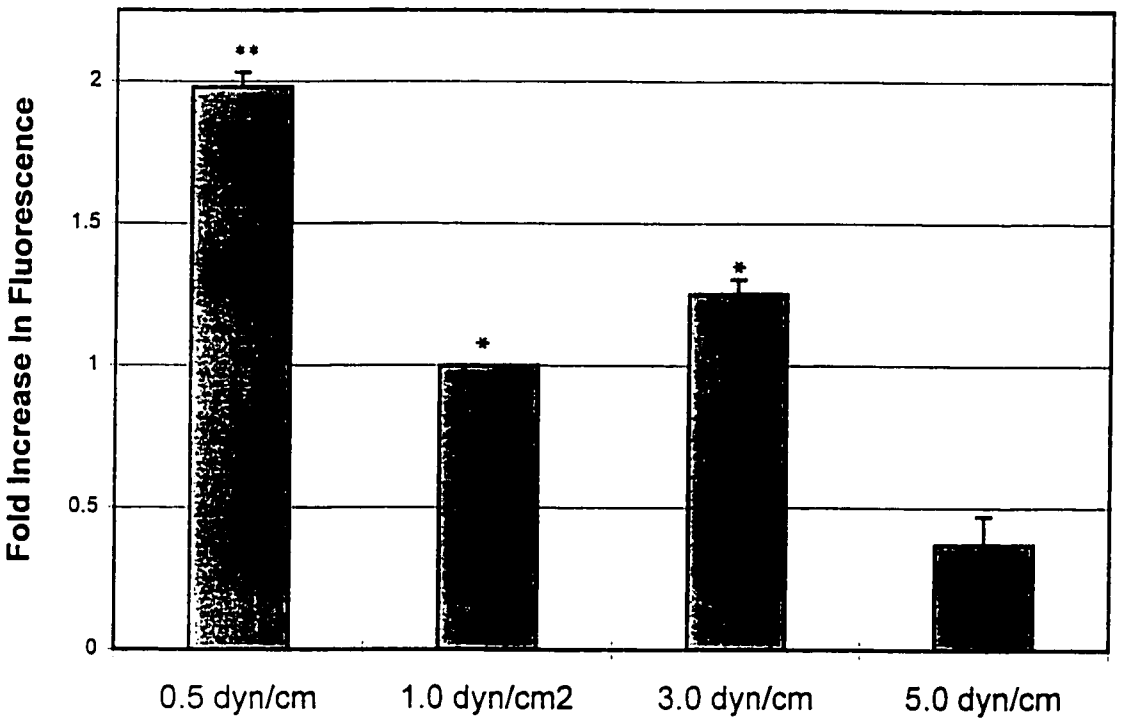


Figure 41. Effect of graded shear stress on adhesion of GzHi tumor cells to 24h cytokine stimulated Ea-hy-926 monolayers. "*"-"**" represent distinct populations with statistical significance ($p<0.05$) between each as determined by Newman-Keuls analysis. Bar graph represents the mean +/- 1 s.d. of 3 repeats.

Chapter 3

DISCUSSION AND CONCLUSIONS

3.1 Introduction

The ability of tumor cells to spread from a primary site of growth to distant organs (metastasis) is the foremost reason for treatment failure in breast cancer patients. Metastasis occurs through a cascade of essential events, each of which is a potential target for anti-metastatic therapy. The extravasation step of metastasis involves binding of complementary adhesion molecules between circulating tumor cells and venular endothelial cells. The breast carcinoma associated mucin, MUC1, has been shown to bind endothelial ICAM-1 in static assays.[34,61] If this adhesive process mediates the extravasation of tumor cells this discovery presents promising treatment strategies. Blockade of this adhesive process would possibly prevent a large majority of breast cancer metastases from occurring as the MUC1 molecule is aberrantly expressed in over 90% of breast cancer patients.[34] Determining if MUC1/ICAM-1 binding mediates tumor cell/endothelial cell attachment in fluid flow conditions is thus extremely important to determining if these molecules should be considered as targets of anti-metastatic therapy in breast cancer.

3.2 Review and Discussion of Experimental Data

3.2.1 Murine Tumor Cells Bind Endothelial Ea-hy-926 via MUC1 and ICAM-1 During Physiological Shear Stress

The expression of human MUC1 on the surface of GzHi murine tumor cells mediates their adhesion to Ea-hy-926 endothelial monolayers during *in vitro* physiological shear stress of 1.0 dyn/cm^2. The presence of the human MUC1 molecule on the murine tumor cells directly correlates with their ability to bind the endothelium, as

MUC1 transfectants (GzHi) adhere more readily than the parental cell line (410.4). Phenotypic analysis of these cell lines indicates that the GzHi cells do not differ from 410.4 in expression of major surface antigens and thus, inadvertent subclone selection was likely not responsible for the difference in adhesion. Thus, increased adhesion of the GzHi cells is due solely to the increase in surface MUC1 expression. The role of MUC1 was definitively proven through inhibition of tumor cell adhesion by pretreatment of the GzHi cells with anti-MUC1. Anti-MUC1 pretreatment reduced adhesion of the GzHi cells to levels virtually identical to those of the untransfected 410.4 cells. It is interesting to note that the antibody used (B27.29) is specific for an immunodominant peptide sequence (PDTRP) found in the extracellular tandem repeat of the MUC1 molecule.[61, 101] This sequence is likely covered by extensive O-linked glycosylation on normal breast epithelium. Decreased glycosylation of MUC1, during tumorigenesis, may then expose this epitope and create an altered MUC1 that has gained the ability to mediate adhesion of breast cancer cells.

The endothelial ICAM-1 molecule was also demonstrated to be specifically involved in the adhesion of the MUC1 transfectant murine tumor cells to endothelium during physiological fluid flow. Endothelial-carcinoma hybrid cells (Ea-hy-926), stimulated for 24 hours with cytokines (TNF-α and IL-1β), had significantly greater surface expression of ICAM-1 compared to cells without stimulation. Tumor cell adhesion to these cells correlated with the ICAM-1 expression as stimulated Ea-hy-926 monolayers supported greater adhesion of the GzHi cells. However, cytokine stimulation alters the expression of many different adhesion molecules, thus ICAM-1 alone may not

be the only molecule responsible for the increase in adhesion seen after cytokine stimulation. The participation of ICAM-1 was demonstrated by the inhibition of adhesion after pretreatment of stimulated Ea-hy-926 with a cocktail of anti-ICAM-1 antibodies (18E3D, 164I, and 164B). A specific epitope on ICAM-1 that mediates binding cannot be determined from these results due to the antibody cocktail but previous studies in static assays have shown that the 18E3D antibody does block MUC1/ICAM-1 binding.[84] It is also known that 18E3D does not effect binding of LFA-1/ICAM-1 and thus is likely not specific to the first amino terminal Ig domain of ICAM-1 (correspondence with ICOS Corp.).

Both the GzHi tumor and Ea-hy-926 endothelial cells were phenotyped for surface expression of complementary adhesion molecules known to be essential for inflammatory cell recruitment. No ligand pairs were found to exist between the GzHi and Ea-hy-926 cells, with the exception of the MUC1 and ICAM-1 molecules. It should be noted that the GzHi cells are of murine origin and that phenotyping was completed using antibodies raised against human antigens. However, any role that murine ligands have in adhesion of GzHi cells is ruled out by the fact that the parental 410.4 cells have decreased adhesion in comparison to the GzHi, proving the role of human MUC1 in adhesion.

3.2.2 Molecular Requirements of ICAM-1-Mediated Adhesion.

GzHi tumor cells bound in fluid flow to crude lysed membrane preparations, made from stimulated Ea-hy-926 cells. This suggests that no active intracellular signaling or extracellular capping of ICAM-1 is required for binding to occur. However, purified rhICAM-1 alone, at ICAM-1 densities similar to the membrane preparation, did

not bind the GzHi cells under shear stress. This suggests that the ICAM-1 molecule has specific molecular requirements which must be met for adhesion to occur. Dimerization of ICAM-1 has been to shown to create higher affinity ligation to the integrins[40,41] and therefore is likely the active form in tumor cell recruitment as well. The rhICAM-1 molecule used in these experiments is present as an ICAM-1 dimer, with two ICAM-1 molecules bound to the Fc portion of an immunoglobulin molecule. This rhICAM-1 dimer has been previously shown to bind the GzHi cells specifically through MUC1 in static conditions.[34,61] This eliminates the possibility that the purified ICAM-1 was not present in a physiologically relevant form.

ICAM-1 and LFA-1 binding cannot occur in fluid flow without prior rolling on selectins.[23] This suggests that MUC1/ICAM-1 binding may also be dependent upon an adhesion cascade to allow for either activation or simply a decrease in rolling velocity of the circulating cells to mediate a stronger bond between MUC1 and ICAM-1. Phenotypic analysis of both GzHi and Ea-hy-926 cells rules out the presence of complementary adhesion ligand pairings which are known to be involved in leukocyte recruitment to endothelium. Thus, an adhesive cascade involving MUC1/ICAM-1 binding might include an unidentified ligand pair.

A second possible explanation for the inability of rhICAM-1 to recruit GzHi tumor cells in fluid flow is that the presence of a lipid bi-layer is needed for the correct ICAM-1 conformation to mediate binding. This theory has been proposed for other tumor associated ligands, such as the carcinoembryonic antigen, which is unable to bind cell ligands when present in soluble vs. membrane bound form.[102] However, in static assays the rhICAM-1 molecule has been demonstrated as a capable ligand for MUC1

expressing cells.[34,61] Thus, the lipid membrane explanation would appear to be unlikely unless the lipid membrane would confer an extremely high affinity to the Ig-fusion protein, rhICAM-1.

3.2.3 Molecular Requirements of MUC1-Mediated Adhesion

MUC1 expressed on murine tumor cells has been demonstrated as a ligand for endothelial ICAM-1 under *in vitro* physiological fluid flow. However, sMUC1 pretreatment of stimulated Ea-hy-926 endothelium could not prevent the recruitment of GzHi cells. Further analysis found that after fluid flow no sMUC1 remained bound to the endothelial monolayers (data not shown) and thus, no inhibition of GzHi adhesion was seen. However, this analysis also revealed that sMUC1 did bind in static conditions which explains previous results from our lab which demonstrated that sMUC1 can prevent GzHi adherence in static assays.[34,61] Thus, for high affinity adhesion of MUC1 to endothelial ICAM-1 specific requirements beyond the soluble protein must be satisfied.

Pretreatment of stimulated Ea-hy-926 endothelial monolayers with MFG, isolated from human breast milk, did not prevent the recruitment of GzHi cells. MFG isolation was accomplished by centrifugation methods which are known to isolate lipid vesicles[103] in the same manner as it was shed from the original epithelium.[104] The presence of MUC1 in MFG was confirmed by Western Blots. The inability of MFG to prevent GzHi recruitment to endothelium would seemingly exclude the presence of a lipid bilayer as a molecular requirement for MUC1 mediated adhesion. However, the source of MFG used in this experiment was a normal, lactating female, where glycosylation of MUC1 is decreased, but still extensive enough to cover cryptic MUC1 epitopes.[105] MUC1/ICAM-1

mediated adhesion to date has been demonstrated exclusively through tumor-derived MUC1 which is known to be underglycosylated.[105] Thus, tumor derived sMUC1, which does not irreversibly bind endothelial ICAM-1, may gain an adhesive conformation if incorporated into a lipid membrane. However, lipid incorporation has been difficult with the human ascites-derived sMUC1 used in these experiments which lacks a transmembrane domain due to cleavage during the purification process.[106]

An adhesive cascade interaction involving multiple ligands is also possible during MUC1-mediated adhesion. The decreased glycosylation of MUC1 in carcinomas has been shown to permit cell-cell ligand interactions of many other surface molecules, including ICAM-1/LFA-1 interactions.[75,107] Though phenotypic analysis of the cells used in the current experiments eliminates the molecules of immune cell trafficking as being part of the adhesion process, it does not exclude numerous other possible ligand pairs. Others have also shown that successful binding under flow conditions may involve redundancy whereby several ligand pairs operate in tandem as necessary but not sufficient adhesion partners for cell recruitment.[108] There is currently no indication what other ligands may be operating in this system.

3.2.4 Effect of Shear Stress on GzHi/Ea-hy-926 Adhesion

The binding mechanism which exists between the GzHi tumor and Ea-hy-926 endothelial cells has shear stress limitations similar to that of well described leukocyte and tumor models. Neutrophils bound to endothelial layers via the adhesion cascade of selectins/carbohydrate ligands and ICAM-1/LFA-1 are only able to adhere at shear stress of less then 3.6 dyn/cm^2 using the parallel plate flow chamber *in vitro* model.[17,23] Beyond

5.0 dyn/cm^2 binding of most cells, tumor or immune, can either no longer be demonstrated or are drastically reduced.[17,23] The GzHi/Ea-hy-926 adhesive mechanism follows this pattern since shear stress above 3.0 dyn/cm^2 would not allow significant adhesion. However, at decreased shear stress, above the physiological limit (1.0 dyn/cm^2), GzHi cells could significantly adhere to stimulated endothelium via the MUC1/ICAM-1 mechanism.

3.2.5 Human Breast Carcinoma Adhesion to HUVECs During Physiological Shear Stress

To determine the role of MUC1/ICAM-1 binding in a more physiologically relevant model the ability of a human breast carcinoma (MCF-7) to adhere to HUVECs was analyzed. MCF-7 tumor cells, expressing significant amounts of MUC1, were found to significantly adhere to 24 hr cytokine-treated HUVECs, with upregulated ICAM-1 expression compared to unstimulated cells, with limited surface ICAM-1 expression. To exclusively implicate the ICAM-1 and MUC1 molecules as mediators of adhesion, antibody inhibition experiments were performed. However, neither anti-MUC1 or anti-ICAM-1 treatment prevented the MCF-7 cells from accumulating on the stimulated HUVECs. As well, anti-ICAM-1 pretreatment of stimulated Ea-hy-926 cells only minimally decreased MCF-7 adhesion. There are two possible explanations for these findings. The first is that the MUC1/ICAM-1 molecules are not significantly involved in the adhesive process demonstrated due to a decreased affinity in this model. This seems unlikely since MUC1/ICAM-1 binding was demonstrated to be significant to the GzHi/Ea-hy-926 model. The second is that MUC1/ICAM-1 binding is active but that

cytokine stimulation of HUVECs upregulates the expression or affinity of a second adhesion molecule that binds an unknown ligand on MCF-7 cells. This redundancy of adhesive ligands would cause accumulation of MCF-7 cells on stimulated HUVECs even in the presence anti-MUC1 or ICAM-1. A similar process has been demonstrated with eosinophils which have multiple ligands for endothelial adhesion molecules and accumulate on endothelium during fluid flow unless all ligand pairs are blocked.[108]

3.3 Theoretical Mechanisms of MUC1/ICAM-1 Mediated Adhesion

Since the soluble MUC1 and immobilized rhICAM-1 are functional as ligands only in static assays, the most probable explanation for the data presented is that an adhesion cascade, involving the MUC1 and ICAM-1 molecules, is responsible for tumor/endothelial cell adhesion during fluid flow. Within this hypothesis arises two possible cascade mechanisms. The first being that the MUC1 and ICAM-1 molecules are ligands for two separate adhesion molecules. This theory is unlikely as static assays have proven ICAM-1 is a ligand for the tumor derived MUC1 molecule.[34] As well, known alternative ligands for the ICAM-1 molecule were not present on the tumor cells used, while ligands for the O-linked carbohydrates present on MUC1 are not present on stimulated endothelial cells. The second possible adhesion cascade includes MUC1 and ICAM-1 as exclusive ligands with a separate, unknown ligand pair also part of the cascade. In this binding mechanism it is probable that the MUC1/ICAM-1 adhesion mediates firm attachment, arrest and accumulation of cells on the endothelium similar to LFA-1/ICAM-1 binding.[22,23] For this reason antibodies specific for MUC1 and ICAM-1 prevent tumor cell accumulation as was seen with the GzHi/Ea-hy-926 adhesion assays in

fluid flow. The second ligand pair would then mediate primary or secondary adhesion and possibly rolling on the endothelial cells. Though MCF-7 accumulation was not prevented on HUVECs with pretreatment of anti-ICAM-1 or anti-MUC1 it is possible that tumor cell accumulation on endothelium occurs via the unknown ligand pair in this model. A similar finding was previously described with eosinophil accumulation via primary binding mechanisms (VCAM-1/VLA-4 and P-selectin/PSGL-1) despite the presence of antibody against the firm adhesion molecules active in the model (β_2 integrins).[108] This eosinophil phenomena was shear stress and cell type dependent.[108] Though neither VCAM-1/VLA-4 or P-selectin/PSGL-1 ligand pairings are present on these tumor models it is possible that other adhesive ligand pairs are present. Furthermore, the fact that anti-ICAM-1 treatment of Ea-hy-926 only minimally prevented MCF-7 adhesion, while significantly reducing GzHi adhesion suggests that the second ligand on the tumor cells is of higher affinity or expression on MCF-7 cells. This would explain why the GzHi/Ea-hy-926 model demonstrates antibody inhibition sensitivity while the MCF-7/HUVEC model does not, even though similar molecules may mediate the adhesive process.

3.4 Clinical Relevance

Recruitment of circulating tumor cells to endothelium via the MUC1/ICAM-1 binding mechanism demonstrates a unique mechanism that possibly mediates extravasation during breast cancer metastasis. Recent publications have demonstrated that MUC1 has characteristics of an adhesive ligand, potentially able to mediate tumor

cell migration. This includes putative intracellular signaling domains[69] as well as linkage to the cytoskeleton.[70] As well, the ICAM-1 molecule is a ligand known to be essential for inflammatory cell extravasation.[109] The signaling function[9,30] and cytoskeletal association[30,31] of ICAM-1 are likely mechanisms involved in creating a permissive endothelial phenotype, allowing circulating cells to migrate through this barrier.

The data presented here suggests the potential for new anti-metastatic therapeutic strategies. One possible treatment strategy would be to block the adhesive interaction through treatment with antibody specific for the MUC1 protein core epitope responsible for binding to ICAM-1. Interestingly, treatment of MUC1 transgenic mice with antibody (SM3), specific for an epitope on the MUC1 tandem repeat, was found to delay spontaneous tumor development and spread.[98] This effect may be attributed to various possible mechanisms. The first being that antibody binding of MUC1 may enhance both humoral and cell-mediated immunity against tumor cells. The second possibility would suggest that extravasation and metastasis were diminished as adhesion to endothelial ICAM-1 was prevented. A final mechanism of inhibition would be through the production of anti-idiotype antibodies against the SM3 antibody.[98] This anti-idiotype specificity may actually allow the antibody to bind the complementary ICAM-1 region on endothelium and block recruitment of tumor cells.

However, recent discoveries on the role of MUC1 in immunity may complicate potential therapeutic strategies. It was recently demonstrated that mitogen-activated T cells of the immune system express MUC1.[86] Thus, treatment with anti-MUC1 may cause impaired immune cell function and/or development as the role of MUC1 on these cells has yet to be determined. The complexity of the immune response is highlighted in

a recent paper investigating the effect liposomal MUC1, which stimulates immune responses in tumor bearing mice. Decreased metastases and prolonged survival were seen only in mice developing a type 1 (T_1) anti-MUC1 response, indicated by increased IgG_2 levels.[110] Mice with increased IgG_1 anti-MUC1 levels had no discernible advantage over control mice as both groups developed lung metastases.[110] No explanation or suggestions were made to explain the different responses in mice.[110] Thus, any advantage that anti-MUC1 treatment provides would presumably be linked to a balance of immune responses and should therefore be approached with caution.

3.5 Conclusions

In recent years it has become increasingly apparent that MUC1 plays a significant role in tumor cell development, spread and survival in human breast carcinoma. MUC1 is expressed at densities up to 10 times that of normal in an underglycosylated form that exposes cryptic protein core epitopes.[34,110] The data presented here clearly demonstrates that this human tumor form of MUC1 can mediate recruitment of murine GzHi cells to the Ea-hy-926 endothelium during *in vitro* physiological shear stress conditions via adhesion with ICAM-1. Adhesion through these molecules represents an active form of tumor cell recruitment which may lead to extravasation through an adhesion molecule cascade mechanism. The MUC1 molecule is thus a target for anti-metastatic therapy that has shown promise in preliminary studies. Future advances in this area of breast cancer treatment will hopefully lead to a blockade of tumor cell recruitment, thus impeding metastases and improving the success rate of existing treatment methods.

Bibliography:

1. National Cancer Institute of Canada, Health Canada. Canadian Cancer Statistics. http://www.cancer.ca/stats/egb010.htm
2. Breast Cancer Statistics. http://www.wwdir.com/survivor/discuss/stats.html
3. Akiyama SK, Olden K, Yamada KM. Fibronectin and integrins in invasion and metastasis. Cancer Metastasis Rev 14: 173, 1995
4. Liotta LA, Rao CN, Barsky SH. Tumor invasion and the extracellular matrix. Lab Invest 49(6): 636, 1983.
5. Liotta LA, Stetler-Stevenson WG. Tumor invasion and metastasis: an imbalance of positive and negative regulation. Cancer Res Suppl 51: 5054s, 1991.
6. Giavazzi R, Foppolo M, Dossi R, Remuzzi A. Rolling and adhesion of human tumor cells on vascular endothelium under physiological flow conditions. J Clin Invest 92: 3038, 1993.
7. Tozeren A, Kleinman HK, Grant DS, Morales D, Mercurio AM. E-selectin mediated dynamic interactions of breast and colonic cells with endothelial-cell monolayers. Int J Cancer 60: 426, 1995.
8. Engelberg H. Actions of heparin that may affect the malignant process. Cancer 85: 257, 1999.
9. Cronstein BN, Weissman G. The adhesion molecules of inflammation. Arthritis Rheum 36: 147, 1993.
10. Carlos TM, Harlan JM. Leukocyte-endothelial adhesion molecules. Blood 84(7): 2068, 1994.

11. Moser R, Schleiffenbaum B, Groscurth P, Fehr J. Interleukin 1 and tumor necrosis factor stimulate human vascular endothelial cells to promote transendothelial passage. J Clin Invest 83: 444, 1989.

12. Butcher EC, Picker LJ. Lymphocyte homing and homeostasis. Science 272: 60, 1996.

13. Frenette PS, Wagner DD. Adhesion molecules - part II: blood vessels and blood cells. Mol Med 335(1): 43, 1996.

14. Bevilacqua MP, Nelson RM. Selectins. J Clin Invest 91: 379, 1993.

15. Crockett-Torabi E. Selectins and mechanisms of signal transduction. J Leuk Biol 63: 1, 1998.

16. Bevilacqua MP, Pober JS, Mendrick DL, Cotran RS, Gimbrone Jr. MA. Identification of an inducible endothelial-leukocyte adhesion molecule. Proc Natl Acad Sci USA 84: 9238, 1987.

17. Lawrence MB, Springer TA. Neutrophils roll on E-selectin. J Immunol 151(11): 6338, 1993.

18. Picker LJ, Kishimoto TK, Smith RA, Warnock A, Butcher EC. ELAM-1 is an adhesion molecule for skin-homing T-cells. Nature 349: 796, 1991.

19. Carlos T, Kovach N, Schwatrz B, Ross M, Newman B, Wayner E, Banjamin C, Osborn L, Lobb R, Harlan J. Human monocytes bind to two cytokine -induced adhesive ligands on cultured endothelial cells: endothelial-leukocyte adhesion molecule-1 and vascular cell adhesion molecule-1. Blood 77: 2266, 1991.

20. Bochner BS, Luscinskas FW, Gimbrone Jr. MA, Newman W, Sterbinsky SA, Derse-Anthony CP, Klunk D, Schleimer RP. Adhesion of human basophils, eosinophils, and neutrophils to interleukin 1-activated human vascular endothelial cells: contributions of endothelial adhesion molecules. J Exp Med 173: 1553, 1991.

21. Wong D, Prameya R, Dorovini-Zis K. In vitro adhesion and migration of T-lymphocytes across monolayers of human brain microvessel endothelial cells: regulation by ICAM-1, VCAM-1, E-selectin and PECAM-1. J Neuropath Experiment Neurol 58(2): 138, 1999.

22. Jones DA, McIntire LV, Smith WC, Picker LJ. A two-step adhesion cascade for T-cell/endothelial cell interactions under flow conditions. J Clin Invest 94: 2443, 1994.

23. Lawrence MB, Springer TA: Leukocytes roll on a selectin at physiological flow rates: distinction from and prerequisite for adhesion through integrins. Cell 65: 859, 1991.

24. Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion: Cell 69: 11, 1992.

25. Rey-Ladino JA, Pyszniak AM, Takei F. Dominant-negative effect of the lymphocyte function-associated antigen-1 β (CD18) cytoplasmic domain on leukocyte adhesion to ICAM-1 and fibronectin. J Immunol 160: 3494, 1998.

26. Wulfing C, Sjaastad MD, Davis MM. Visualizing the dynamics of T cell activation: intracellular adhesion molecule 1 migrates rapidly to the T cell/B cell interface and acts to sustain calcium levels. Proc Natl Acad Sci USA 95(11): 6302, 1998.

27. Hmama Z, Knutson KL, Herrera-Velit P, Nandon D, Reiner NE. Monocyte adherence induced by lipopolysaccharide involves CD14, LFA-1, and cytohesin-1. Regulation by Rho and phosphatidylinositol 3-kinase. J Biol Chem 274(2): 1050, 1999.

28. Vedder NB, Harlan JM. Increased surface expression of CD11b/CD18 (Mac-1) is not required for stimulated neutrophil adherence to cultured endothelium. J Clin Invest 81: 676, 1988.

29. Philips MR, Buyon JP, Winchester R, Weissmann G, Abramson SB. Up-regulation of the iC3b receptor (CR3) is neither necessary nor sufficient to promote neutrophil aggregation. J Clin Invest 82: 495, 1988.

30. van de Stolpe A, van der Saag PT. Intercellualar adhesion molecule-1. J Mol Med 74: 13, 1996.

31. Carpén O, Pallai P, Staunton DE, Springier TA. Association of intercellular adhesion molecule-1 (ICAM-1) with actin-containing cytoskeleton and α-actinin. J Cell Biol 118(5): 1223, 1992.

32. Diamond MS, Staunton DE, Marlin SD, Springer TA. Binding of the integrin Mac-1 (CD11b/CD18) to the third immunoglobulin-like domain of ICAM-1 (CD54) and its regulation by glycosylation. Cell 65: 961, 1991.

33. Rosenstein Y, Park JK, Hahn WC, Rosen FS, Bierer BE, Burakoff SJ. CD43 a molecule defective in Wiskott-Aldrich syndrome, binds ICAM-1. Nature 354: 233, 1991.

34. Regimbald LH, Pilarski LM, Longenecker BM, Reddish MA, Zimmerman G, Hugh JC. The breast mucin MUC1 as a novel adhesion ligand for endothelial intercellular adhesion molecule 1 in breast cancer. Cancer Res 56: 4244, 1996.

35. Knorr R, Dustin ML. The lymphocyte function-associated antigen 1 I domain is a transient binding module for intercellular adhesion molecule (ICAM-1) and ICAM-3 in hydrodynamic flow. J Exp Med 185(5): 719, 1997.

36. Marlin SD, Springer TA. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). Cell 61: 813, 1987.

37. Etienne S, Adamson P, Greenwood J, Strosberg AD, Cazaubon S, Couraud PO. ICAM-1 signaling pathways associated with Rho activation in microvascular brain endothelial cells. J Immunol 161: 5755, 1998.

38. Yamada A, Hara A, Inoue M, Kamizono S, Higuchi T, Itoh K. β_2-integrin mediated signal up-regulates counter receptor ICAM-1 expression on human monocytic cell line THP-1 through tyrosine phosphorylation. Cell Immunol 178: 9, 1997.

39. Bassi V, De Riu S, Feliciello A, Altomonte M, Allevato G, Rossi G, Fenzi GF. Intercellular adhesion molecule-1 is upregulated via the protein kinase C pathway in human thyroid carcinoma cell lines. Thyroid 8(1): 23, 1998.

40. Miller J, Knorr R, Ferrone M, Houdei R, Carron CP, Dustin ML. Intercellular adhesion molecule-1 dimerization and its consequences for adhesion mediated by lymphocyte function associated-1. J Exp Med 182: 1231, 1995.

41. Reilly PL, Woska JR, Jeanfavre DD, McNally E, Rothlein R, Bormann B. The native structure of intercellular adhesion molecule-1 (ICAM-1) is a dimer. J Immunol 155: 529, 1995.

42. Furie MB, Naprstek BL, Silverstein SC. Migration of neutrophils across monolayers of cultured microvascular endothelial cells. J Cell Sci 88: 161, 1987.

43. Greenwood J, Wang Y, Calder VL. Lymphocyte adhesion and transendothelial migration in the central nervous system: the role of LFA-1, ICAM-1, VLA-4 and VCAM-1. Immunology 86: 408, 1995.

44. Bianhci E, Bender JR, Blasi F, Pardi R. Through and beyond the wall: late steps in leukocyte transendothelial migration. Immunol Today 18(12): 586, 1997.

45. Huber AR, Weiss SJ. Disruption of the subendothelial basement membrane during neutrophil diapedesis in an in vitro construct of a blood vessel. J Clin Invest 83: 1122, 1989.

46. Durieu-Trautmann O, Chaverot N, Cazaubon S, Strosberg AD, Couraud PO. Intercellular adhesion molecule 1 activation induces tyrosine phosphorylation of the cytoskeleton-associated protein cortactin in brain microvessel endothelial cells. J Biol Chem 269 (17): 12536, 1994.

47. Goetzl EJ, Banda MJ, Leppert D. Matrix metalloproteinases in immunity. J Immunol 156: 1, 1996.

48. Romanic AM, Madri JA. The induction of 72-kD gelatinase in T cells upon adhesion to endothelium is VCAM-1 dependent. J Cell Biol 125 (5): 1165, 1994.

49. Xia M, Leppert D, Hauser SL, Sreedharan SP, Nelson PJ, Krensky AM, Goetzl AJ. Stimulus specificity of matrix metalloproteinase dependence of human T cell migration through a model basement membrane. J Immunol 156 (1): 160, 1996.

50. Leppert D, Waubant E, Galardy R, Bunnett NW, Hauser SL. T cell gelatinases mediate basement membrane transmigration in vitro. J Immunol 154 (9): 4379, 1995.

51. Kawaguchi T, Nakamura K. Analysis of the lodgment and extravasation of tumor cells in experimental models of hematogenous metastasis. Cancer Metastasis Rev 5: 77, 1986.

52. Luzzi KJ, MacDonald IC, Schmidt EE, Kerkvliet N, Morris VL, Chambers AF, Groom AC. Multistep nature of metastatic inefficiency. Am J Pathol 153 (3): 865, 1998.

53. Brenner W, Langer P, Oesch F, Edgell CS, Wieser RJ. Tumor cell-endothelium adhesion in an artificial venule. Anal Biochem 225: 213, 1995.

54. Jahroudi N, Greenberger JS. The role of endothelial cells in tumor invasion and metastasis. J Neurooncol 23: 99, 1995.

55. Kojima N, Shiota M, Sadahira Y, Handa K, Hakomori S. Cell adhesion in a dynamic flow system as compared to static system. J Biol Chem 267 (24): 17264, 1992.

56. Radinsky R. Molecular mechanisms for organ-specific colon carcinoma metastasis. Eur J Cancer 31A (7/8): 1091, 1995.

57. Sawada T, Ho JJL, Chung Y, Sowa M, Kim Y. E-selectin binding by pancreatic tumor cells is inhibited by cancer sera. Int J Cancer 57: 901, 1994.

58. Alexandroff AB, McIntyre CA, Porter JC, Zeuthen J, Taub DD. Sticky and smelly issues: lessons on tumour cell leucocyte trafficking, gene and immunotherapy of cancer. Br J Cancer 77 (11): 1806, 1998.

59. Price EA, Coombe DR, Murray JC. Beta-1 integrins mediate tumour cell adhesion to quiescent endothelial cells in vitro. Br J Cancer 74: 1762, 1996.

60. Goetz DJ, El-Sabban ME, Hammer DA, Pauli BU. Lu-ECAM-1-mediated adhesion of melanoma cells to endothelium under conditions of flow. Int J Cancer 65: 192, 1996.

61. Kam JL, Regimbald LH, Hilgers JHM, Hoffman P, Krantz MJ, Longenecker BM, Hugh JC. MUC1 synthetic peptide inhibition of intercellular adhesion molecule-1 and MUC1 binding requires six tandem repeats. Cancer Res 58: 5577, 1998.

62. Gendler SJ, Spicer AP. Epithelial mucin genes. Annu Rev Physiol 57: 607, 1995.

63. Ho SB, Shekels LL, Toribara NW, Kim YS, Lyftogt C, Cherwitz DJ, Niehans GA. Mucin gene expression in normal, preneoplastic, and neoplastic human gastric epithelium. Cancer Res 55: 2681, 1995.

64. Hilkens J, Wreschner MJL, Vos HL, Litvinov S. Cell membrane-associated mucins and their adhesion-modulating property. Trends Biochem Sci 17: 1066, 1992.

65. Devine PL, McKenzie IF. Mucins: structure, function and association with malignancy. Bioessays 14(9): 619, 1992.

66. Patton S, Gendler SJ, Spicer AP. The epithelial mucin, MUC1, of milk, mammary gland, and other tissues. Biochim Biophys Acta 1241: 407, 1995.

67. Spicer AP, Rowse GJ, Lidner TK, Gendler SJ. Delayed mammary tumor progression in Muc-1 null mice. J Biol Chem 270(50): 30093, 1995.

68. Zrihan-Licht S, Vos HL, Baruch A, Elroy-Stein O, Sagiv D, Keydar I, Hilkens J, Wreschner DH. Characterization and molecular cloning of a novel MUC1 protein, devoid of tandem repeats, expressed in human breast cancer tissue. Eur J Biochem 224: 787, 1994.

69. Wreschner DH, Zrihan-Licht S, Baruch A, Sagiv D, Hartman M, Smorodinsky N, Keydar I. Does a novel form of the breast cancer marker protein, MUC1, act as a receptor molecule that modulates signal transduction? Adv Exp Med Biol 353: 17, 1994.

70. Parry G, Beck JC, Moss L, Bartley J, Ojakian GK. Determination of apical membrane polarity in mammary epithelial cell culture: the role of cell-cell, cell-substratum, and membrane cytoskeleton interaction. Exp Cell Res 188: 302, 1990.

71. Fontenot JD, Tjandra N, Bu D, Ho C, Montelaro RC, Finn OJ. Biophysical characterization of one-, two-, and three-tandem repeats of human mucin (muc-1) protein core. Cancer Res 53: 5386, 1993.

72. Kam JLM. MUC1 synthetic peptide inhibition of ICAM-1 and MUC1 binding is dependent on the number of tandem repeats. University of Alberta: M.Sc. Thesis, 1998.

73. Ligtenberg MJL, Buijs F, Vos HL, Hilkens J. Suppression of cellular aggregation by high levels of episialin. Cancer Res 52: 2318, 1992.

74. Wesseling J, van der Valk SW, Vos HL, Sonnenberg A, Hilkens J. Episialin (MUC1) overexpression inhibits integrin-mediated cell adhesion to extracellular matrix components. J Cell Biol 129: 255, 1995.

75. Magarian-Blander J, Ciborowski P, Hsia S, Watkins SC, Finn OJ. Intercellular and intracellular events following the MHC-unrestricted TCR recognition of a tumor specific peptide epitope on epithelial antigen MUC1. J Immunol 160(7): 3111, 1998.

76. van de Wiel-van Kemenade E, Ligtenberg MJL, de Boer AJ, Buijs F, Vos HL, Melief CJM, Hilkens J, Figdor CG. Episialin (MUC1) inhibits cytotoxic lymphocyte-target cell interaction. J Immunol 151(2): 767, 1993.

77. Zhang KE, Sikut R, Hansson GC. A MUC1 mucin secreted from a colon carcinoma cell line inhibits target cell lysis by natural killer cells. Cell Immunol 176: 158, 1997.

78. Suwa T, Hinoda Y, Makiguchi Y, Takahashi T, Itoh F, Adachi M, Hareyama M, Imai K. Increased invasiveness of MUC1 cDNA-transfected human gastric cancer MKN74 cells. Int J Cancer 76: 377, 1998.

79. Yamamoto M, Bharti A, Li Y, Kufe D. Interaction of the DF3/MUC1 breast carcinoma-associated antigen and β-catenin in cell adhesion. J Biol Chem 272(19): 12492, 1997.

80. Li Y, Bharti A, Chen D, Gong J, Kufe D. Interaction of glycogen synthase 3β with the DF3/MUC1 carcinoma-associated antigen and β-catenin. Mol Cell Biol 18 (12): 7216, 1998.

81. Mareel M, Boterberg T, Noë V, van Hoorde L Vermeulen S, Bruyneel E, Bracke M. E-cadherin / catenin / cytoskeleton complex: a regulator of cancer invasion. J Cell Physiol 173: 271, 1997.

82. Zhang KE, Baekstrom D, Hansson GC. A secreted mucin carrying sialyl-Lewis a from colon carcinoma cells binds to E-selectin and inhibits HL-60 cell adhesion. Int J Cancer 59(6): 823, 1994.

83. Pandey P, Kharbanda S, Kufe D. Association of the DF3/MUC1 breast cancer antigen with the Grb2 and the SOS/Ras exchange protein. Cancer Res 55: 4000, 1995.

84. Regimbald LHJ. The role of MUC1 in vascular adhesion: an essential step in breast cancer metastasis. University of Alberta: M.Sc. Thesis, 1996.

85. Agrawal B, Krantz MJ, Reddish MA, Longenecker BM. Cancer-associated MUC1 mucin inhibits human T-cell proliferation which is reversible by IL-2. Nat Med 4(1): 43, 1998.

86. Agrawal B, Krantz MJ, Parker J, Longenecker BM. Expression of MUC1 mucin on activated T cells: implications for a role of MUC1 in normal immune regulation. Cancer Res 58(18): 4079, 1998.

87. Agrawal B, Gendler SJ, Longenecker BM. The biological role of mucins in cellular interactions and immune regulation: prospects for cancer immunotherapy. Mol Med Today 4(9): 397, 1998.

88. Hilkens J, Wesseling J, Vos Hl, Storm J, Boer B, van der Valk SW, Mass MCE. Involvement of the cell surface-bound mucin, episialin/MUC1, in progression of human carcinoma's. Biochem Soc Trans 23: 822, 1995.

89. McIntire LV, Eskin SG. Mechanical and biochemical aspects of leukocyte interactions with model vessel walls. *White cell mechanics: basic science and clinical aspects*. New York: Alan R Liss Inc.: p.209, 1984.

90. Lawrence MB, McIntire LV, Eskin SG: Effect of flow on polymorphonuclear leukocyte/endothelial cell adhesion. Blood 70(5): 1284, 1987.

91. Cutnell JD, Johnson KW. *Physics 3rd Edition.* New York: John Wiley and Sons, Inc.: p.308, 1995.

92. Morigi M, Zoja C, Figliuzzi, Foppolo M, Micheletti G, Bontempelli M, Saronni M, Remuzzi G, Remuzzi A. Fluid shear stress modulates surface expression of adhesion molecules by endothelial cells. Blood 85 (7): 1696, 1995.

93. Davies PF. How do vascular endothelial cells respond to flow? News Physiol Sci 4: 22, 1989.

94. Frangos JA, McIntire LV, Eskin SG. Shear stress induced stimulation of mammalian cell metabolism. Biotechnology and Bioengineering 32: 1053, 1988.

95. Lawrence MB, Smith CW, Eskin SG, McIntire LV. Effect of venous shear stress on CD18-mediated neutrophil adhesion to cultured endothelium. Blood 75(1): 227, 1990.

96. Thornhill MH, Li J, Haskard DO. Leucocyte endothelial adhesion: a study comparing human umbilical vein endothelial cells and the endothelial cell line Ea-hy-926. Scand J Immunol 38: 279, 1993.

97. Reiber AJ, Marr HS, Comer MB, Edgell CS. Extent of differentiated gene expression in the human endothelium-derived Ea.hy926 cell line. Thromb Hemost 69(5): 476, 1993.

98. Girling A, Bartkova J, Burchell J, Gendler S, Gillett C, Taylor-Papadimitriou J. A core protein of the polymorphic epithelial mucin detected by the monoclonal antibody SM-3 is selectively exposed in a range of primary carcinomas. Int J Cancer 43: 1072, 1989.

99. Graham RA, Burchell JM, Taylor-Papadimitiou J. The polymorphic epithelial mucin: potential as an immunogen for a cancer vaccine. Cancer Immunol Immunother 42: 71, 1996.

100. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(259): 680, 1970.

101. Reddish MA, Helbrecht N, Almeida AF, Madiyalakan R, Suresh MR, Longenecker BM. Epitope mapping of mAb B27.29 within the peptide core of the malignant breast carcinoma - associated mucin antigen coded for by the human MUC1 gene. J Tumor Marker Oncol 7: 19, 1992.

102. Kammerer R, von Kleist S. The carcinoembryonic antigen (CEA) modulates effector-target cell interactions by binding to activated lymphocytes. Int J Cancer 68: 457, 1996.

103. Shimuzu M, Yamauchi K. Isolation and characterization of mucin-like glycoprotein in human fat globule membrane. J Biochem 91(2): 515, 1982.

104. Sekine H, Ohno T, Kufe DW. Purification and characterization of a high molecular weight glycoprotein detectable in human milk and breast carcinomas. J Immunol 135(5): 3610, 1985.

106. Ligtenberg MJ, Kruijshaar L, Buijs F, van Meijers M, Litvinov SV, Hilkens J. Cell-associated episialin is a complex containing two proteins derived from a common precursor. J Biol Chem 267(9): 6171, 1992.

107. Bohm CM, Mulder MC, Zennadi R, Notter M, Scmitt-Graff A, Finn OJ, Taylor-Papadimitriou J, Stein H, Clausen H, Riecken EO, Hanski C. Carbohydrate recognition on MUC1-expressing targets enhances cytotoxicity of a T cell subpopulation. Scand J Immunol 46: 27, 1997.

108. Patel KD. Eosinophil tethering to interleukin-4-activated endothelial cells requires both P-selectin and vascular cell adhesion molecule-1. Blood 92(10): 3904, 1998.

109. Del Maschio A, Zanetti A, Corado M, Rival Y, Ruco L, Lampugnani MG, Dejana E. Polymorphonuclear leukocyte adhesion triggers the disorganization of endothelial cell-to-cell adherens junctions. J Cell Biol 135(2): 497, 1996.

110. Samuel J, Budzynski WA, Reddish M, Ding L, Zimmermann GL, Krantz MJ, Koganty RR, Longenecker BM. Immunogenicity and antitumor activity of a liposomal MUC1 peptide-based vaccine. Int J Cancer 75: 295, 1998.