CROSSTALK BETWEEN COAGULATION AND RECEPTOR MEDIATED TUMOR CELL ADHESION TO ENDOTHELIUM UNDER HYDRODYNAMIC SHEAR.

A Thesis in Bioengineering by Tugba Ozdemir

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Abstract

Coagulation is a programmed cascade of events known to be playing a pivotal role in immune surveillance as well as the blood clot formation. Although, coagulation is known to be crucial for immune surveillance, this process is considered to be linked with tumor metastasis in recent studies. Since the primary coagulation initiator thrombin generation needed to be activated by inflammatory cytokines and tissue factors it is not surprising to face increased coagulation parameters in tissue factor expressing tumor microenvironment and innate immune system components such as platelets and PMNs (polymorphonuclear neutrophils). During their travel in hydrodynamic flow, tumor cells need to adhere in order to prevent from anoikis and spread through distant organs. On the other hand, tumor cells do not have appropriate receptors to adhere to the endothelium by themselves. They need to anchor themselves through different mediators such as the components of host immune system which are capable of travelling freely in the body. For this reason PMNs are known to be the best candidates for metastatic tumor cells. PMNs are immune cells which have the capability to adhere to endothelium via specific Selectins and Integrins. They are capable of not only resisting high shear conditions of hemodynamic flow but also transmigrating through the endothelial wall and travel to distant organs in which they are needed. In this study, we are seeking to model the above proposed hydrodynamic tumor microenvironment with creating a steady state hydrodynamic flow in vitro by putting all the possible components together and investigate the adhesion mechanism of highly metastatic tumor cells to endothelium.
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Chapter 1
Background and Significance

1.1.1. Background:

1.1.2. Melanoma Metastasis as a Disease

Melanoma is the deadliest form of skin cancer and the United States has experienced a dramatic increase in the number of melanoma cases over the past few decades. According to the American Cancer Society, the incidence rate for melanoma is 100,000 people each year. The leading cause of melanoma death is the metastasis to distant organs and new tumor formation. Following the primary tumor formation and angiogenesis, tumor cells tend to escape to blood circulation as a next step of their progression (Gassmann, 2008).

It is generally accepted that a large number of tumor cells enter the circulation, but a low percentage will successfully undergo metastasis to target organs. Main reasons for the elimination of tumor cells can be listed as mechanical shear forces and cell mediated cytotoxicity within the circulation which leads to anoikis (apoptosis of tumor cells due to lack of adhesion). In order to protect themselves from anoikis and the circulation dependent cytotoxicity, tumor cells must adhere to the endothelium. After successfully adhering to endothelium, tumor cells can either proliferate or undergo transmigration to survive and form new colonies (Steeg, 2006).
In the circulation potential metastatic target organs presents a set of pro- and anti-metastatic stimuli regulating the faith of metastatic organ colonization. Mechanical shear stress, anoikis and cell mediated cytotoxicity destruct most of the tumor cells from the circulation can be listed as anti- metastatic stimuli. Adhesion and further extravasation is the fundamental interactions tumor cells experience that can be classified as pro-metastatic stimuli.

Tumor cell adhesion to the post capillary venules is tightly regulated with organ specific availability of adhesion molecules on tumor cells, endothelium and the other cellular components of the tumor microenvironment. The secretion of adhesive and migratory paracrine signals can initiate the cascade of events leading to melanoma metastasis. During cancer metastasis, tumor cells prefer certain tissues as their hosts and form secondary colonies for the progression of the disease (Nicolson, 1988). The different preference to different organs shows that cancer metastasis is a highly regulated and multi-step process and it should be evaluated regarding these characteristics. The metastatic cascade can be explained by a “seed and soil” hypothesis by Stephen Paget in late 1880s (Paget, 1889). The hypothesis suggests the organ colonization and growth of a tumor would initiate from the primary tumor and clutter to other tissues by the help of circulation (Fig.1.1.).
Fig.1.1. Melanoma Metastasis Process (O’Hayre, 2008).
The consecutive steps of metastasis are potentially regulated by organ specific manner. The seed and soil hypothesis has formed a basis for several concepts and further theories. The “adhesion theory” focuses on the role of organ specific adhesive interactions of tumor cells in the capillary bed of metastatic tissues. After being survived from the high circulation forces tumor cells enter to post capillary venules of host organs and initiate primary tumor formation with the support of several chemical and biological mechanisms (Fidler, 2003). Our primary focus in this study is based on the adhesive interactions tumor cells experience in their microenvironment.
1.1.3. Coagulation and Cancer

The relationship between cancer and thrombosis roots from the observations of Professor Armand Trousseau who pointed the idiopathic venous thromboembolism (VTE) during the autopsies of cancer patients with severe metastasis. Two key players have shown to play important roles in the link between cancer metastasis and coagulation; (1) the protease enzyme Thrombin and (2) Tissue Factor, the primary initiator of the coagulation cascade and a transmembrane receptor which leads to several cellular mitogenic processes such as growth and differentiation of the cells (Rickles, 2003).

Coagulation and hemostasis is tightly regulated by several positive and negative feedback mechanisms due to the kinetic nature of the process. Basically, coagulation is regulated by two main pathways. The intrinsic (contact) pathway can be triggered by conversion of Factor VII into Factor VIIa due to conformational change as a result of contact to a surface. The second mechanism that triggers coagulation is the Tissue Factor Pathway. Tissue Factor Pathway starts from the primary cellular initiator (Tissue Factor membrane protein) of coagulation by binding to Factor VII and turning it into Factor VIIa and further initiate coagulation (Figure 1.2.). As a part of our project, we will also be focusing on the mechanisms relating with the Tissue Factor pathway.
Circulating tumor cells with metastatic phenotype are known to express high levels of tissue factor, a potent activator of the Factor VII dependent coagulation cascade. Coagulation can be triggered easily by TF and can result in thrombin generation and further fibrin cleavage. Thrombin is the essential player of hemostasis generated upon tissue injury. Thrombin generation depends on complex enzymatic mechanism by which, when coagulation is triggered, thrombin first generated, and then subsequently inactivated. In the mechanism of thrombin generation three essentially different types of activation can be observed; thrombin activation, inactivation and modulation of reaction velocity. The balance between thrombin generation and inactivation and the reaction velocities take place in haemostatic regulation and by far formation of DIC (disseminated intravascular coagulation) (Hemker, 1995). DIC is a common observation in metastatic cancer patients and regulates tumor microenvironment by elevating plasma coagulation proteins.
When coagulation is triggered, the substrate molecules (fibrinogen, membrane receptors, e.g.) cleaved by thrombin, determines the extent of the thrombolytic response. If the substrate levels are not exhausted, the number of molecules converted by thrombin is proportional to both the concentration of the enzyme and the time that it can act. The thrombin generation mechanism is regulated by many positive and negative feedback mechanisms and its kinetics shows a show a nonlinear behavior. At some point parameters may vary without changing the output very much, on the other hand, small changes in one or more constituent activities may very large effect on the output (Hemker, 1995).

Thrombin regulates several mechanisms besides from converting fibrinogen into fibrin by cleaving it at Aα16R-17G. Besides converting fibrinogen into fibrin, thrombin regulates cellular behavior independent of clotting by activating G-protein-coupled PAR receptors. Thrombin also assembles adhesion molecules (i.e. P-selectin) to the endothelial surface and also increase the expression levels of adhesion receptors (ICAM-I, VCAM-I) to support tumor cell adhesion (Rickles, 2003). Apart from its cellular effects, thrombin increases several bioactive cleavage fragments of fibrinogen that contribute to tumorigenesis. Relevance of tissue factor dependent coagulation and increased fibrin formation in tumor microenvironment affect the adhesion process and further regulate extravasation (Rickles, 2001). Elevated levels of fibrin shown to be a prognostic significance with advanced stages of colorectal, lung and breast cancer (Rickles, 2003).
1.1.4. Fibrinogen&Fibrin

Fibrinogen is a glycoprotein having two sets of three polypeptide chains, Aα, Bβ, and γ. Linked together by 29 disulfide bonds having a mass of 340 kDa. Fibrinogen is 45 nm when elongated and two outer globular domains formed by many α-helical coiled coil domains. Fibrin formation is initiated after thrombin cleavage of the A fibrinopeptides, which are located at the N-terminal ends of the fibrinogen Aα chains. This cleavage exposes binding sites in the central domain that are constitutively exposed in γ chain (Konstantopoulous, 2009).

Fibrin (ogen) molecule has binding sites for several proteins, such as fibronectin and von Willebrand factor; integrins and integrin ligands, such as the platelet αIIbβ3, the leukocyte αMβ2, αvβ3 and ICAM-I (Fig.1.3.). Fibrinogen play roles in several biological events, including homeostasis/thrombosis, inflammation, wound healing and cancer metastasis. It is believed that platelet/fibrin (ogen) clots surrounding tumor cells may protect them from immunologic and physiologic stresses and facilitate their localization to the post capillary venules (Palumbo, 2005).
Fig. 1.3. Fibrinogen Molecule and cryptic binding regions for integrin molecules (Sigma Corp.)
1.1.5. **Tumor Cell Adhesion Mechanisms.**

A successful tumor cell adhesion is a multi step process and may require cross linking cells and plasma proteins to form tighter bonds. Hemodynamic flow orchestrates blood cell and EC interaction through the cell collision, alterations of forces on cell-cell adhesive bonds and regulates the cellular signaling. Due to their similar origin, tumor cells share similar adhesive receptors with the endothelial cells (Glinsky, 2005). This fact prevents direct adhesion of tumor cells to the endothelium. Adhesion to the post-capillary venules of the target organs is highly controlled by the organ-specific match of adhesion molecules (especially integrins) on tumor cells, endothelium and extracellular matrix. Epithelial and mesenchymal tumor cells require adhesion dependent mechanisms for survival. The most effective way for a circulating tumor cells to escape from anoikis is to form adhesive interactions with the host tissue. It is important to note that tumor cell adhesion is limited to capillary beds of target organs rather than the large vessels.

Initial arrest and adhesion to host organ endothelium is the crucial step in hematogenous metastasis. Selectins and inter-cellular adhesion molecule-I (ICAM-I) are the main adhesion receptors present on endothelial cells. Tumor cells must form adhesive interactions with those receptors in order to firmly adhere and start the extravasation process. For arrest, tumor cells can take advantage of polymorphonuclear neutrophils (PMNs) to enhance their metastatic potential. Melanoma cells express ICAM-I and $\alpha_v\beta_3$. ICAM-I enables tumor cells to adhere to PMNs (Slattery, 2005). This heterotypic interaction allows tumor cells to adhere to the endothelium. In addition to PMNs fibrin
(ogen) is also having cryptic regions for both ICAM-I and α,β3 and it can further enforce PMN mediated tumor cell adhesion to the endothelium.

1.1.6. Cell Adhesion under Shear Flow.

One of the important points in adhesion theory is that the tumor cell adhesion appears to be limited to the capillary bed of potential metastatic target organs with the suitable microenvironment for their survival. This theory led us to further investigate the components of tumor microenvironment and which mechanistic events play a role in melanoma metastasis. In addition to suitable receptor pairings, a tumor cell population adhesion event is regulated by the shear flow and the forces generated in that environment. A cell of radius R forms one or more bonds with counter receptors on the endothelium, fluid shear stresses exert a torque (T) and a drag force (Fx) on the cell. The net hydrodynamic force is balanced by force (Fb) that stresses the bonds (Rinker, 2001) (Fig 1.4.).

Shear rate on the other hand is dependent on the flow rate and the geometry of the flow field. Shear rate modulates the transport of the cells to the other regions and other cells. Transport of the cells modulates the cell-cell collision and the contact time between those cells.
**Fig. 1.4.** The model for fluid-induced forces acting on a cells rolling across the endothelial monolayer in laminar shear flow (Rinker, 2001).
1.1 Significance:

Considerable evidence supports that the primary initiator of the coagulation cascade, TF (Tissue Factor), has a role in cancerous processes and tumor metastasis. TF may influence metastasis by the production of proteins that may create a suitable environment for metastasis, especially at the adhesion level. Circulating tumor cells with metastatic phenotype are known to express high levels of tissue factor, a potent activator of Factor VII dependent coagulation cascade. Relevance of tissue factor dependent coagulation and increased fibrin formation in tumor microenvironment would affect the adhesion process and further regulate extravasation. Fibrin is the resultant of coagulation with having the capability of binding a wide range of cellular receptors, including two of the leukocyte receptors Mac-1 and LFA-1 and αvβ3 and ICAM-1 on both tumor cells and endothelial cells. In this study, we will investigate the possible mechanisms that support the tumor adhesion to the endothelium. Because fibrin is a crosslinker for tumor cells, PMNs and the endothelium the cross reactions between these cell pairings in the presence of fibrin carry utmost significance.
Chapter 2
Triggered Coagulation in Tumor Microenvironment

2.1 Introduction:

A better understanding of the role of thrombin in metastasis will need knowledge of several mechanisms that are known to support malignancy. Previous studies demonstrated that fibrinogen is the most abundant substrate of thrombin which both supports tumor cell adhesive events as well as the angiogenic capability of a given tumor type (Palumbo, 2005). Thrombin is potent activator of angiogenesis. Fibrinogen is also known to leak through tumor vessels. Upon leaking through the vessel, fibrinogen then binds to specific receptors on inflammatory cells and tumor cells and also it is cleaved by thrombin that is generated in the local tumor microenvironment. The generated fibrin that is deposited around the neoangiogenic vessels acts as cement for tumor cells to form new colonies and further grow (Rickles, 2003, Chest).

Elevated fibrin levels in tumor microenvironment are associated with overexpressed Tissue factor (TF) transmembrane protein in tumor cells (Ngo, 2007) and it has been well documented in previous literature. Thrombin, the critical enzyme in coagulation and it is also taking important roles in inflammatory events. Thrombin induces neutrophil adhesion to endothelium, P- and E-Selectin expression and specific chemokine secretion. Another important effect that is associated with thrombin is the elevated ICAM-I (CD54) levels. Here, we showed that thrombin treatment of HUVEC (Human Umbilical Vein Endothelial Cells) affected ICAM-I levels and it is closely associated with treatment time (Kaplanski, 1998).
Melanoma and many metastatic cells express TF membrane protein on their surfaces and it is successfully shown by many investigators (Muller, 1992, Ngo, 2007) that TF on tumor cells membrane is closely correlated with increased metastasis (Rickles, 2003). TF binds to Factor VII with a very high affinity (K_a < 10 pM). The generation of thrombin starting with this cascade follows Michaelis-Menten enzyme kinetics, in which the relative velocity of the reaction depends hyperbolically on the available substrate concentration (Hatchcock, 2006).

The role of enzyme kinetics on surfaces under static conditions to show that the relative “steady-state” velocity of product formation on a surface (V/V_max) can be expressed in (Kobayashi, 1974):

\[
\frac{V}{V_{\text{max}}} = \frac{[S]}{K_m + [S]}
\]

In which, V is the reaction velocity, [S] is the substrate concentration and K_m is the Michaelis-Menten rate constant. Under given circumstances, thrombin generation in a tumor microenvironment would also follow a hyperbolic trend that would further affect the dynamics of the events that are linked with thrombin exposure. Both in vitro and in vivo experimental evidence have shown that melanoma metastasis and coagulation cascades are positively correlated. Another important role thrombin plays is it cleaves fibrinogen into fibrin, controlling the last step of clot formation (Rickles, 2003).
This chapter examines how melanoma and interactions with the endothelial cells induce changes in coagulation properties in tumor microenvironment. In the first part, three cell lines of melanoma will be studied: WM35, Lu1205 and HUVEC. Of the three, Lu1205 is classified as highly metastatic while WM35 is non-metastatic; HUVEC cells are also known not to induce coagulation if there are no inflammatory signals. The capability of a cell to trigger coagulation by generating thrombin is investigated.

The study will also focus on the adhesion molecule expression changes on endothelium in response to elevated and prolonged thrombin stimulant conditions. It is widely believed that thrombin is a potent inflammatory signal on endothelium to induce expression of adhesion molecules, specifically ICAM-I. HUVEC are studied to investigate the changes in the primary adhesion receptor in response to thrombin treatment.

### Table 2-1

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<tr>
<td>WM35</td>
<td>-</td>
<td>Non metastatic tumor cell line</td>
<td>Wistar Institute</td>
</tr>
<tr>
<td>Lu1205</td>
<td>+++</td>
<td>Metastatic variant of WM791 melanoma</td>
<td>(Juhasz et al., 1993)</td>
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**Table 2-1.** Comparisons of metastatic potentials of melanoma cells used in the study. Each “+” represents a relative measure of metastatic potential, “-” represents absence of metastatic potential.
2.2 Experimental Preparation and Procedures

2.2.1 Cell Culture

Human melanoma cell line, WM35, was provided by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA). The cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Biosource, Inc, Camerillo, CA) supplemented with 10% by volume fetal bovine serum (FBS; Atlanta Biologicals, GA). Another human melanoma cell line, Lu1205 (provided by Dr. Gavin P Robertson, Penn State Hershey Medical Center, Hershey, PA) is cultured in Dulbecco’s Modified Eagle’s Medium F12 (DMEM F12), also supplemented with 10% FBS. Prior to each experiment, cells were detached with 0.05% trypsin/EDTA (Invitrogen, Carlsbad, CA) and washed twice with fresh medium. After then, cells were suspended in fresh media and allowed to recover for 1 hr while being rocked at a rate of 8 rpm at 37 °C.

Human umbilical vein endothelial cells (ATCC, America Type Culture Collection, Manassas, VA) were grown in Ham’s F12k media (Biofluids Inc, Gaithersberg, MD). The HUVECs are supplemented in the media by 10% FBS, 10ug/ml endothelial growth supplement (VWR, Westchester, PA) and 100ug/ml heparin (Sigma Chemical Co, St Louis, MO). All media contain 100 units/ml of penicillin/streptomycin (Biofluids, Inc) and all cells were maintained under the conditions of 37 °C in an incubator with 5% carbon dioxide.
2.2.2 Cell Lysis

To collect the cell lysate, the cell culture media was aspirated and 50-100μl of cell lysis buffer (Sigma, St Louis, MO) was added to the base of the culture dish. A rubber scraper was used to scrape the cells together with the lysis buffer. The dish is then placed on ice for 30 minutes. The lysate is collected into a micro-centrifuge tube and spun at 10,000 RPM for 5 minutes to pellet any cell debris. The debris-free lysate is then obtained for freeze storage or for further experiments.

2.2.3 Plasma and Thrombin.

Platelet-poor (citrated) plasma (PPP) is used both in traditional hematology experiments and in studies of coagulation/anticoagulation (Zhou, 2005). Recalcified PPP is completely coagulation competent, requiring no additional proteins or phospholipids to recover clotting potential. Human platelet-poor plasma (hPPP, citrated) was prepared from outdated (within 2 days of expiration) lots obtained from the M.S. Hershey Medical Center Blood Bank. This work was performed with a single pooled lot of plasma aliquoted into 15 ml polypropylene tubes (Falcon, Becton Dickinson) and frozen at -20 ºC until use. Consistent results are observed with this plasma over the 1 year time span of experimentation. Experience has shown that different lots of plasma yield quantitatively different but qualitatively similar results. Human a-thrombin (Sigma-Aldrich) solutions were freshly prepared before each use by dissolution in phosphate-buffered saline (PBS, Sigma-Aldrich) from 0 to 1 NIH unit/ml.
2.2.4 Coagulation Time Assays.

The basic protocol for coagulation-time assays used in this work has been described in details elsewhere (Zhou, 2005) and applied in this work in two basic variations: thrombin titration (TT), and thrombin-cell lysate co-titrations (COT). In each variation, 0.5 ml of thawed plasma equilibrated with ambient temperature was transferred into 2 ml polystyrene tubes (VWR) and diluted with sufficient PBS to bring the final total liquid volume to 1 ml; including all additives which varied according to the assay. In TT experiments, serially increasing volumes between 0 and 1 U/ml of thrombin were added to each tube in the titration series. In all cases, coagulation was induced by recalcification with 0.1 ml of 0.1M CaCl2 and contents were mixed on a slowly turning hematology mixer (Roto-shake Genie, Scientific industries, Inc.). Coagulation time CT after recalcification was observed by a distinct change in fluid like rheology to gel formation, allowing determination of the endpoint of the coagulation process to within 10 s or so.

2.2.5 Statistical Analysis

Data analysis was performed using Minitab software (State College, PA). Significance was defined as p< 0.05 in and unpaired t-test analysis, and repeated measures ANOVA.
2.3 Results

2.3.1 Thrombin titration of human plasma

In order to sensitively compare the human PPP coagulation with reproducibility, the ‘thrombin titration’ (TT) experiments done in which the plasma coagulation time CT of recalcified plasma induced by increasing concentrations of human alpha thrombin [T]. Fig 2.1. shows the plasma coagulation time in response to increasing thrombin concentrations. It can be seen from the plot that the trend shows an exponential decay approaching to ‘zero minute’ time at increasing thrombin levels. The results agrees with the rate of an enzyme reaching to a plateau after the saturation concentrations explained in Michaelis Menten kinetics.

![Graph showing plasma coagulation in response to thrombin treatment.](chart.png)

**Fig. 2.1.** Plasma coagulation in response to thrombin treatment.
2.3.2 Melanoma Cell Induced Plasma Coagulation.

The procoagulant ability of the cell lysates were analyzed with plasma coagulation assay. The activity is associated with thrombin concentration being generated by comparing it with the thrombin titration curve (Fig. 2.1.). Tumor cells known to induce coagulation by membrane bound TF protein. It is widely known that endothelial cells do not express TF under normal conditions. Our results indicate that melanoma cells (WM35) with non metastatic properties are not a strong procoagulant as well as the endothelial cells (HUVEC).

In contrast, melanoma cells with highly metastatic capability triggered coagulation significantly. Fibrin generation and increased thrombin concentration are important inflammatory promoters that help tumor cells to elicit metastasis. The tumor cell types with aggressive behavior can induce coagulation more than the non metastatic cell lines suggesting that increased procoagulant function is a property of metastatic cells and would facilitate the progression of the disease by promoting thrombin generation and subsequent fibrin cleavage. Fibrin is also investigated in this study as a crosslinker supporting tumor cell adherence to endothelium under inflammatory conditions.
Functional intervention in the blood coagulation pathway at the level of TF and thrombin affects experimental melanoma metastasis (Fischer, 1995). TF activity on the extracellular side of the melanoma cells initiates the cascade of events required to generate thrombin in vitro. Thrombin can accomplish variety of biological effects such as, the activation of platelets and endothelial cells, generation of fibrin. In this study we observed that thrombin generation is significantly depend on the metastatic capacity of a given melanoma cell line (Fig. 2.2) In many cell types, thrombin known to evoke different biological responses including $[\text{Ca}^{2+}]$ mobilization, mitogenic signals and induction of adhesion receptor expression.

![Graph](image)

**Fig 2.2.** Functional procoagulant potential of different cell lines. Procoagulant ability was quantified by the time to clot human plasma. Human melanoma cell lines WM35 and Lu1205, HUVEC cell lysates were used to investigate the ability to produce thrombin. The data were then used to estimate how much thrombin being produced by the given cell type.
In summary, our results demonstrated that one of the characteristic property that metastatic tumor microenvironment has is the increased procoagulant capacity. HUVEC, did not show a significant rise in procoagulant capacity at rest (not induced by inflammatory cytokines, such as TNF-alpha). Endothelial cells known not to trigger coagulation when they are not induced, however upon inflammation endothelial cells express TF to trigger coagulation. Lu1205 cells that are known with their high metastatic and aggressive nature was able to trigger coagulation significantly. That would explain the increased coagulant parameters around tumor lesions in vivo (Ngo, 2007). Tumor cells with known aggressive behavior can induce thrombin generation and further fibrin cleavage in vitro. Fibrin is the potential crosslinker for tumor cells supporting their arrest and further firm adhesion in vitro, and the scope of this study is fibrin mediated tumor cell interaction with endothelium and host immune cells.
2.3.3 Effect of Thrombin on Adhesion Receptor Expression on Endothelium.

Thrombin is involved in coagulation, but it also plays an important role affecting cellular properties. *In vitro*, inflammation can be described by a multistep adhesive interaction between leukocytes and the endothelium. Adhesive interactions during inflammation include the interactions between the different families of molecules such as selectins, integrins and the Ig superfamily (Kaplanski, 1998). Thrombin is previously shown to induce P- and E-selectin expression on HUVECs and may play a role in the first step of adhesion. The last step of adhesion would be characterized as the strong adhesive interactions with the help of integrin molecules. In this step, one of the primary endothelial adhesion molecule ICAM-I (CD54) is of great interest not only for its ability to form a tight bond between leukocytes and endothelium, but it also serves as a fibrin receptor providing a chance to tumor cells to interact with vessel wall with elevated fibrin(ogen) in tumor microenvironment.

In this part of the study, we focused on the ability of HUVEC cells to response to thrombin in the environment. In addition, we investigated the time dependent response of HUVEC endothelial cells to thrombin. This is important to understand the dynamic regulation of events leading to ICAM-I expression on endothelial surface. We have found that thrombin is a strong inflammatory stimulant on HUVEC cells even stronger than the TNF-alpha under same incubation conditions (Fig. 2.3).
Fig 2.3. Thrombin induces CD54 expression on HUVECs (Flow cytometry analysis). HUVECs were cultured for 3 hours, 6 hours, 12 hours and 24 hours in culture medium (upper panels), in the presence of 2 U/mL of thrombin or in the presence of TNF-alpha. After cell trypsinization, CD54 expression was studied by FACS analysis (1 representative experiment among 3) (Courtesy of Pu Zhang)
2.4 Discussion

For metastasis to occur, tumor cells (TC) must leave the primary tumor and enter the circulatory system, they extravasate through the endothelial lining of a blood vessel to proliferate in another organ. While in the circulatory system, TCs can remain as single cells or form homo- or heterotypic emboli with other cell types (e.g., leukocyte or platelets), but they must survive the shear forces encountered in the blood flow (Nguyen, 2004; Shevde and Welch, 2003). TCs can arrest in the microvasculature due to size restriction or become tethered to the vascular endothelium (EC) using a variety of surface adhesion molecules as described previously. Experiments presented here suggest that hemodynamic forces, heterotypic cell populations, endogenously secreted inflammatory cytokines within a tumor microenvironment, and PMN-melanoma adhesion play a complex role in the recruitment of metastatic cancer cells to the endothelium.

The activation of coagulation and successful metastasis has long been associated with each other. Tissue factor is a transmembrane protein often expressed on the inflamed tissues to activate coagulation via activating Factor VII. One of the characteristic of metastatic phenotype in melanoma cells is the increased levels of membrane tissue factor levels and previous studies successfully showed that it is positively correlated with the metastatic phenotype in melanoma (Mueller, 1992). Once tumor cells enter the microvascular capillaries surface Tissue factor might be triggering the coagulation via the extrinsic pathway of blood coagulation.
Another important finding recently been shown is the effect of physiological flow on TF activation of coagulation (Okorie, 2008). Diamond et al. clearly showed even at concentrations of femtomolar level, surface TF protein strongly triggers coagulation. However this concentration has not the same effect under static conditions. These data urged us to investigate for a clearer picture for the link between the fibrin generation in vivo and the membrane bound TF levels on metastatic tumor cells. It is very fascinating to study the dynamic regulation of thrombin in tumor microenvironment towards elevating procoagulant parameters in tumor microenvironment. These interactions might create a suitable environment for circulating tumor cells to successfully adhere and transmigrate through the host tissues.
Chapter 3

The Effect of Fibrin(ogen) on PMN Facilitated Melanoma Adhesion

3.1 Introduction:

During their travel in the circulation, tumor cells experience hydrodynamic shear forces that prevent them from successfully adhering to the endothelium and further transmigrate to distant organs. Cell adhesion is the critical step towards metastasis and tumor cell use several mechanisms to adhere to the endothelium. In inflammation processes, extensive leukocyte extravasation is a characteristic determinant. A sequential multistep model explains the mechanism that sustains the leukocytes even under high shear conditions. In this process, leukocytes first weakly attach the vessel wall and start rolling over the endothelial lining, after then leukocytes start forming stronger bonds between β2 integrins and endothelial adhesion molecules to form firm adhesive bonds.

Previous in vitro studies have shown that neutrophils (PMNs) facilitate melanoma cell extravasation by intercellular adhesion molecule-1 (ICAM-1) and β2-integrin bridging (Liang, 2008). However, the extent of adhesive interactions supporting tumor cell capture by PMNs and other components of the blood that might be supporting this process have not been shown yet to get a more mechanistic understanding about the metastasis problem.
3.2 Materials and Methods:

3.2.1 Antibodies and Reagents

Mouse IgG anti-human monoclonal antibodies (mAbs) against αvβ3 (anti-CD51/61, clone 23C6) and ICAM-1 (clone BBIG-I1) were purchased from R&D systems (Minneapolis, MN). Mouse anti-human mAbs against LFA-1 (anti-CD11a) and Mac-1 (anti-CD11b) were purchased from Invitrogen (Carlsbad, CA). N-formyl-methionyl-leucyl-phenylalanine (fMLP), Fg (Fraction I, type I: from human plasma), GPRP (Gly-Pro-Arg-Pro amide), TRITC (Tetramethylrhodamine isothiocyanate Isomer R), and BSA (bovine serum albumin) were purchased from Sigma (St Louis, MO). Fibronectin was obtained from VWR. Human serum albumin (HSA) was purchased from Calbiochem (La Jolla, CA). Thrombin bovine (269,300U/g) was purchased from MP Biomedicals (Solon, Ohio).

3.2.2 Cell Culture and PMN preparation

Lu1205 (provided by Dr. Gavin Robertson, Penn State University Hershey Medical Center, Hershey, PA) and WM35 (provided by Dr. Meenhard Herlyn, Wistar Institute, Philadelphia, PA) melanoma cells were grown in DMEM/F12 (Dulbecco’s Modified Eagle Medium Nutrient Mixture F12) and RPMI1640 (GIBCO; Carlsbad, CA), respectively, supplemented with 10% FBS (BioSource; Carlsbad, CA). Prior to each experiment, Lu1205 cells were detached with 0.05% trypsin/EDTA (Invitrogen) and washed twice with fresh medium. The cells were then suspended in fresh media and allowed to recover for 1h while being rocked at a rate of 8rpm at 37°C. In some receptor blocking experiments,
Lu1205 cells were pre-treated with respective functional blocking antibody, e.g., anti-αvβ3 (5µg/ml); anti-hICAM-1 (5µg/ml) for 30min at 37ºC, before an onset of assays.

PMN preparation was previously described (Liang, 2005). Following The Pennsylvania State University Institutional Review Board (IRB)-approved protocols (no. 19311), we collected fresh human blood from healthy adults by venipuncture. PMNs were isolated using a Ficoll-Hypaque (Histopaque, Sigma) density gradient as described by the manufacturer and kept at 4°C in Dulbecco's phosphate-buffered saline (DPBS) containing 0.1% HSA for up to 4h before an experiment.

3.2.3 Preparation of Fibrin Solution

Soluble Fibrin (Fn) was made freshly in ion-free DPBS prior to each experiment. To produce soluble Fn monomers and prevent coagulation, thrombin cleavage of Fg was initiated in the presence of 4mM GPRP-NH₂ following a previously used protocol (Biggerstaff, 2006). In order to investigate the rates of Fn production, thrombin concentrations were varied, while keeping a chosen dose from soluble fibrinogen and GPRP in selected experiments. For example, to make 1ml of Fn solution, 120µl Fg (25mg/ml) and 84µl of GPRP (24mM) were mixed with either 2.7µl, 5.5µl or 207µl thrombin (10U/ml, 269,300U/g) immediately before incubation at 37ºC for 5min. Then, a two-fold concentrated Fn stock solution was subsequently mixed with cell suspension (containing tumor cells and/or PMNs) 1sec before experiments at 1:1 ratio to reach a desired Fg, thrombin, GPRP and cell concentrations.
3.2.4 Statistical analysis

Data were obtained from at least three independent experiments and expressed by the means. Statistical significance of difference between means was determined by using Student’s t-test or ANOVA. Tukey’s test was used for post hoc analysis for ANOVA. Probability values of P<0.05 were chosen as statistical significance.

3.2.5 Parallel Plate Flow Chamber:

The most common device in adhesion studies is the parallel-plate system, in which two glass plates are separated by a gasket that defines the flow field. Medium is pumped through the chamber with a syringe pump. The apparatus is mainly constructed then the construct can be placed on an inverted microscope stage to view and do the analysis of adhesive events. The critical dimension is chamber height, which is determined by gasket thickness, in our experiments 12.7 µm. Shear stress is determined with the formula;

\[ \tau = \frac{6Q\mu}{wh^2} \]

Where \( \tau \) is shear stress, \( \mu \) is the fluid dynamic viscosity, \( Q \) is the volumetric flow, \( w \) is chamber width and \( h \) is chamber height. Parallel-plate flow chamber analysis is a specialized in vitro assay system used for studying melanoma, neutrophil and endothelial adhesive interactions under shear stress conditions similar those imparted on the surface of a post-capillary venule. This technique helps us to analyze adhesion events in real time for onscreen visualization as well as record cell rolling in video format. Cell adhesion
parameters, such as rolling frequency, shear resistance and binding/tethering efficiency, are calculated and exported to an Excel spreadsheet and subjected to statistical analysis. Adhesion experiments were performed to understand how PMN-Lu1205 cell aggregates and individual cells adhere to the EC monolayer in the presence or absence of soluble fibrin (ogen) in the media. Soluble fibrin play a role on both tumor cell endothelium interactions and the PMN facilitated tumor cell adhesion events. The experimentally determined tethering frequency, $F_t$, was the number of PMNs that adhered to the EC monolayer per unit time and area, including both rolling and firmly arrested cells. This frequency was normalized by cell flux to the surface to compensate for the different concentration of cells passing the same area of the substrate at different shear rates. At higher shear rates, a higher concentration of cells would pass the endothelium and have higher opportunity to adhere. This normalization followed the procedure cited in Rinker et al’s (2001) work based on equations from Munn et al. (1994).
Fig. 3.1. Parallel Plate System Setup (modified from Wiese, 2009).
All of our cell-cell collision and adhesion experiments were performed in a parallel-plate flow chamber (Glycotech, Rockville, MD) mounted on the stage of a phase-contrast optical microscope (Diaphot 330, Nikon, Japan). A syringe pump (Harvard Apparatus, South Natick, MA) was used to generate a steady flow field in the flow chamber. A Petri dish (35 mm) with a confluent EI cell monolayer (acting as a ligand-binding substrate) was attached to the flow chamber. All experiments were performed at 37°C. The field of view was 800 μm long (direction of the flow) by 600 μm. The focal plane was set on the EI monolayer.

The flow chamber was perfused with appropriate media over the EI monolayer for 2-3 min at a shear rate of 40 s⁻¹ for equilibration before the introduction of a predetermined concentration (1 × 10⁶ cells/ml) of PMNs and Lu1205. PMNs were stimulated with 1 μM fMLP for 1 min before the perfusion into parallel-plate flow chamber. After allowing PMNs and Lu1205 cells to interact with the EI monolayer at a shear rate of 40 s⁻¹ for 2 min, shear rate then adjusted to the experimental range of 62.5-200 s⁻¹ and kept constant for 5 min. Experiments were performed in triplicate and analyzed off-line.
Fig.3.2. Chamber assembly and media perfusion.
3.3 Results

3.3.1. sFg/sFn mediated Tumor Cell Adhesion on Endothelium.

Under laminar flow conditions tumor cells interact with endothelium with very weak adhesive interactions. Melanoma cells express considerable levels of ICAM-I and $\alpha_v\beta_3$ on the cell membranes (Table 3.1). Fibrinogen has specific binding regions for both of the receptors and could link the tumor cells to the endothelial ICAM-I by forming $\alpha v \beta 3$ - sFg/sFn-ICAM-I and ICAM-I-sFg/sFn-ICAM-I bonds in vitro. One of the first observations in this study was the increased Tumor cell endothelium interactions in the presence of sFg/sFn in the perfusion media. The observed phenomenon was quantified in terms of cell retention times for 1 sec, 3 sec and 5 sec respectively to better measure the strength and the kinetics of cell-cell interactions (Fig. 3.4.).

The observed phenomenon is consistent in high shear as well as the low shear conditions. Although under high shear conditions the distinct role of sFn as a stronger crosslinker may form from the other conditions. Long term (possibly involving integrins) tumor cell retention shows an increasing trend in both shear rates (62.5 and 200 sec$^{-1}$). The significance between the inflammatory and normal fibrin levels are both tested and analyzed. In this case, we can conclude that long term retention is facilitated by plasma fibrin levels especially in inflammatory conditions and the increase in retention is driven by plasma proteins(with respect to control case).
### Table 3.1. Comparison of Melanoma cell adhesion receptor expressions (Courtesy of Pu Zhang)

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Geometric Mean Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WM35 (Low metastatic)</td>
</tr>
<tr>
<td>Control IgG</td>
<td>2.8 ± 0.14</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>10.5 ± 0.07</td>
</tr>
<tr>
<td>αvβ3</td>
<td>16.34 ± 0.5</td>
</tr>
</tbody>
</table>

*Values are geometric mean fluorescence intensities ±SEM (n > 3).*
Fig. 3.3. Tumor cell endothelium interactions in the presence of sFg/sFn in the environment. sFg/sFn serves as a crosslinker bridging endothelial ICAM-I to the melanoma cell ICAM-I/αvβ3.
Fig. 3.4. Effects of sFg/sFn on Tc arrest on endothelium under different shear rates (A. 62.5, B. 200 sec\(^{-1}\) respectively). *\(P < 0.05\) compared with the Lu1205 and sFg case in the same time interval. Values are mean ± SEM for \(N \geq 3\).
3.3.2. sFg/sFn mediated PMN adhesion to Endothelium.

Fibrinogen increased PMN adhesion by providing additional bonds between Mac-1 and endothelial ICAM-I. The extent of adhesion in the presence of fibrin is shown to overcome fibrinogen. This is because of the exposed binding sites upon cleavage of fibrinogen by thrombin (Konstantopuolos, 2009).

![Graph showing PMN adhesion](image)

**Fig.3.5.** Effects of sFg/sFn on PMN arrest on endothelium under different shear rates.

*P < 0.05 compared with the Lu1205 and sFg case at the same time interval. Values are mean ± SEM for N ≥ 3.
3.3.3. PMN facilitated Tumor Cell Adhesion in the Presence of sFg/sFn.

Tumor cell retention for the 1 sec time period introduced tumor population to the environment and we clearly saw that the number is elevated when treating the cells with plasma proteins. The observed phenomenon is consistent under high shear as well as the low shear conditions. Under high shear conditions the distinct role of sFn as a stronger cross-linker arises from the other conditions.

Long term (5 sec) tumor cell arrest under both low and high shear rates increased in the presence of sFg/sFn. However fibrin contribution signifies under high shear compared to sFg, which indicates that the coagulation product fibrin has more strength to keep an individual tumor cell closer to endothelium under high shear rates. These results suggest that long term retention is dominated by fibrin especially under high shear rates with respect to fibrinogen and also no plasma protein conditions. Due to the fact that fibrin has a significant contribution to long term TC-EC interactions we can consider $\alpha_v \beta_3 \sim$ sFn$\sim$ ICAM-I or ICAM-I$\sim$ sFn$\sim$ ICAM-I might be stronger than $\alpha_v \beta_3 \sim$sFg$\sim$ICAM-I or ICAM-I$\sim$sFg$\sim$ICAM-I in vitro. TC$\sim$PMN collision would result in favor of tumor cell adhesion to endothelium through a tethered PMN, this phenomena would be defined with adhesion efficiency. Our results clearly showed that plasma sFg/sFn levels are highly correlated with prolonged thus successful tumor cell aggregation with PMNs. This would lead us to think what the mechanism behind increased adhesion efficiency is and how this would contribute to the tumor cell metastasis formation.
PMNs help tumor cell binding to ICAM-I via its β2 integrins and other studies suggest that fibrin(ogen) has multiple binding sites for both Mac-I and ICAM-I as well as αvβ3 on tumor cells which would be an extra supporting force under hydrodynamic opposing forces against tumor cell-PMN receptor-ligand interaction forces (Liang, 2005). The primary goal of this study is how would fibrin elevate the binding strength as well as the receptor-ligand kinetics of TC-PMN-EC and build a supporting model of these observed phenomena to further build pharmacological antagonists to overcome this process.

The chance of successful adhesion for melanoma cells in order to undergo metastasis is a highly complex process. Addition of sFg/sFn to tumor microenvironment might be facilitating the tumor cell adhesion efficiency by increasing the interactions between tumor cells and PMNs. It is previously shown by our group that CD11a/CD18, CD11b/CD18 on PMNs can bind to tumor ICAM-I under hydrodynamic shear conditions. Moreover, αvβ3 arises as a secondary interaction molecule between tumor cell and PMNs as forming αvβ3-sFg/sFn-CD11b/CD18, which might enhance tumor cell adhesion efficiency via PMNs under hydrodynamic shear conditions. Our preliminary data shows that the presence of sFg/sFn enhances tumor cell adhesion efficiency via PMNs significantly under both high and low shear rates.
Adhesion efficiency of Lu1205 was calculated by dividing the number of TCs arrested on ECs by the number of PMN-TC collisions. TC-PMN collisions were those that occurred near EC monolayer surface during flow assay:

\[
\text{Adhesion Efficiency} = \frac{\text{No. of Tumor Cell PMN aggregations under given time}}{\text{No of total TC PMN collisions}}
\]
Fig.3.6. Effects of sFg/sFn on Lu1205 Adhesion efficiency on endothelium under different shear rates (A. 62.5, B. 200 sec\(^{-1}\) respectively). *\(P < 0.05\) compared with the Lu1205 case at the same time interval.
Short term aggregations between PMNs and melanoma cells show a similar trend showing that the coagulation proteins plays a role in short term aggregation between PMNs-TCs. The contribution of sFg/sFn in short term aggregation processes is both similar in both low and the high shear case. Short term aggregations would be an intermediate cell-cell interaction process. We can even see the sFg/sFn contribution to this process and this would be due to role of fibrin(ogen) as an introductory molecule between two colliding cells.

The median or intermediate term PMN-TC aggregations follow a similar trend at the 1 sec retention time. This also gives us an idea about the limit of short term vs. long term interactions between cell groups. In my experiments and results we saw that 1 sec and 3 sec time periods shows a similar trend but the long term interactions behavior distinctive than the other two.

One of the most important conclusions we can come up with high shear long term adhesion is the effect of fibrin. This effect also present in low shear values but significant when the shear rate is increased and this gives strength to the TC-PMN interaction. The results are statistically significant both with control case and the other cases.
3.3.4. Functionally Blocking sFg/sFn Receptors and its Effect on Tumor Adherence.

Blocking functional receptors on tumor cells shows the role of ICAM-I and $\alpha_v\beta_3$ to the tumor cell retention in the presence of fibrin(ogen) process. ICAM-I is the primary linker molecule when there is fibrin(ogen). Our results clearly suggests that fibrin(ogen) act as a cross-linker between Tumor cells and endothelium, however the consistency of the retentions on endothelium is not prolonged. In order for tumor cells to undergo radial locomotion they need resistance to shear for longer time periods. Our findings suggest that the tumor cell retention on endothelium is more of a tethering than a firm adhesion (Figure 3.7).

Blocking of functional adhesion receptors for ICAM-I and $\alpha_v\beta_3$ respectively dramatically affected the Tumor cell host interactions. The short term aggregation of tumor cell and PMN is regulated mainly by ICAM-I on TC however 3 sec and the 5 sec longer aggregation times are driven mostly by both of the receptors (ICAM-I and $\alpha_v\beta_3$ on TC) but mainly on $\alpha_v\beta_3$. This information suggest us fibrin mediated TC-PMN aggregations are mainly regulated by $\alpha_v\beta_3$ and ICAM-I receptors on TC in the presence of sFg/sFn. Fibrin is the mediator enforcing the interactions between the adhesion receptor ICAM-I on endothelium and $\alpha_v\beta_3$ and ICAM-I receptors on TC (Figure 3.8)
Fig. 3.7. Effects of blocking functional antibodies of Tumor cell on the arrest on endothelium. * for the $P < 0.05$ compared with the shown case at the same time interval. Values are mean ± SEM for $N \geq 3$. 
Fig. 3.8. Effects of blocking functional antibodies of Tumor cell adhesion efficiency on endothelium. * for the $P < 0.05$ compared with the shown case at the same time interval. Values are mean ± SEM for $N \geq 3$. 
3.4. Discussion.

Tumor cell adhesion to endothelium under hydrodynamic shear is a multistep process. Previous results from our group showed the contribution of PMNs on Tumor cell adherence to the endothelium. However, PMNs are not the only components of the blood. Consistent evidence show the critical role of coagulation proteins to metastasis. In this study we aimed to investigate the correlation between the PMN facilitated Tumor cell capture and the main coagulation player fibrin(ogen). Fibrin(ogen) regulated tumor cell endothelium interaction both on the tumor cell endothelium interaction side and the PMN tumor cell interaction site. The overall outcome for the fibrinogen presence in tumor microenvironment shows a tendency to be on the favor of tumor cell.

Tumor cells use fibrin(ogen) as a crosslinker between the ICAM-I and $\alpha_1\beta_3$ on Tumor cell side to the ICAM-I on the endothelium. Moreover, tumor cell arrest with PMN with tight interactions are also showed an increasing pattern in the presence of fibrin(ogen). In conclusion, besides being a critical clotting agent for the homeostasis, fibrinogen can act as a crosslinker to bridge tumor cells to vascular endothelium and adhered PMNs.
3.5. Future Research Directions.

The association between coagulation and tumor cell adhesion is a very interesting topic including a very wide range of receptor-receptor interaction and binding kinetics. In this study we showed fibrinogen is a mediator of ICAM-I, $\alpha_\text{v}\beta_3$ and Mac-1 enhancing the binding ability of tumor cell to vascular endothelium. However, the kinetics of binding relationships of this process still remains elusive. The association between the kinetics under hydrodynamic flow is the next interesting step waiting to be investigated.
Chapter 4

Studying Fibrin Mediated Adhesive Interactions at Single Bond Level

4.1. Introduction.

Melanoma cell adhesion process under hydrodynamic flow requires certain amount of receptor ligand interactions and integrins play a pivotal role by maintaining stable adhesion. PMNs are shown to facilitate tumor cell adhesion by forming firm adhesive interactions by the interactions between PMN $\beta_2$ integrins and ICAM-I on both tumor cells and the endothelium. Soluble fibrin, however contributes to direct tumor cell endothelium interactions as well as supporting PMN facilitated Tumor cell adhesion phenomena resulting an overall increase in tumor–cell host interactions. Increased tumor cell host interactions may lead to increased metastasis since tumor cells must use adhesion to migrate through endothelium (Hoskins, 2006)

ICAM-I and $\alpha_v\beta_3$ is the most abundant adhesion receptor expressed on melanoma cell surface adhesion receptors that can form adhesive interactions to sFn. It can be assumed that the adhesive bonds formed between tumor cell ICAM-I and/or $\alpha_v\beta_3$-sFn-endothelial ICAM-I as a chemical reaction. It is reasonable to question and study the rates of binding and unbinding events. The rate of receptor ligand binding is associated with both time of receptor ligand interaction and the intrinsic parameters of the molecules.
The interactions between tumor cell integrins with the endothelial ICAM-I governed by sFn have not been studied extensively and estimating the kinetic parameters in these interactions is the focus of this study.

**What kind of information needed?**

The adhesion of Lu1205 cells to the endothelium via sFn is mediated mainly the intrinsic dissociation rates and can be determined by the method used by Vitte et al (Vitte, 2004). Mainly, each adhesion was considered to be due to the formation of an individual bond. After this assumption and using the elementary kinetics theory, the rate of dissociation of a bond can be determined using

\[
B(t) = B(t = 0)e^{-K_{off}t}
\]

In this equation, \(K_{off}\), the intrinsic dissociation rate constant for a bond breakage, \(t\) is time, and \(B(t)\) is the number of bonds at time, \(t\). The number of bonds at time zero is known and the number of bonds at a certain amount of time can be determined by plotting the lifetime of the bonds, rate constant can be determined from the above formula. The unbinding plot can be made by plotting \(\ln[B(t)]\) vs time to visualize the trend of tumor cell dissociation.
Figure 4.1. Receptor mediated melanoma endothelium interactions by sFn.
4.2 Materials and Methods:

4.2.1 Antibodies and Reagents

Mouse IgG anti-human monoclonal antibodies (mAbs) against \( \alpha_v \beta_3 \) (anti-CD51/61, clone 23C6) and ICAM-1 (clone BBIG-I1) were purchased from R&D systems (Minneapolis, MN). Mouse anti-human mAbs against LFA-1 (anti-CD11a) and Mac-1 (anti-CD11b) were purchased from Invitrogen (Carlsbad, CA). N-formyl-methionyl-leucyl-phenylalanine (fMLP), Fg (Fraction I, type I: from human plasma), GPRP (Gly-Pro-Arg-Pro amide), TRITC (Tetramethylrhodamine isothiocyanate Isomer R), and BSA (bovine serum albumin) were purchased from Sigma (St Louis, MO). Fibronectin was obtained from VWR. Human serum albumin (HSA) was purchased from Calbiochem (La Jolla, CA). Thrombin bovine (269,300U/g) was purchased from MP Biomedicals (Solon, Ohio).

4.2.2 Cell Culture

Lu1205 (provided by Dr. Gavin Robertson, Penn State University Hershey Medical Center, Hershey, PA) melanoma cells were grown in DMEM/F12 (Dulbecco’s Modified Eagle Medium Nutrient Mixture F12) supplemented with 10\% FBS (BioSource; Carlsbad, CA). Prior to each experiment, Lu1205 cells were detached with 0.05\% trypsin/EDTA (Invitrogen) and washed twice with fresh medium. The cells were then suspended in fresh media and allowed to recover for 1h while being rocked at a rate of 8rpm at 37\(^\circ\)C. In receptor blocking experiments, Lu1205 cells were pre-treated with respective functional blocking antibody, e.g., anti-\( \alpha_v \beta_3 \) (5\( \mu \)g/ml); anti-hICAM-1 (5\( \mu \)g/ml) for 30min at 37\(^\circ\)C, before an onset of assays.
Fibroblast L-cells that had been transfected to express human E-selectin and ICAM-1 (provided by Dr Scott Simon, UC Davis, CA) are cultured following the method described previously (Simon, 2000). EI cells express ICAM-I comparable to human umbilical vein endothelial cells (HUVEC).

4.2.3 Preparation of Fibrin Solution

Soluble Fn was made freshly in ion-free DPBS prior to each experiment. To produce soluble Fn monomers and prevent coagulation, thrombin cleavage of Fg was initiated in the presence of 4mM GPRP-NH₂ following a previously used protocol (Biggerstaff, 2006). In order to investigate the rates of Fn production, thrombin concentrations were varied, while keeping a chosen dose from soluble fibrinogen and GPRP in selected experiments. For example, to make 1ml of Fn solution, 120µl Fg (25mg/ml) and 84µl of GPRP (24mM) were mixed with either 2.7µl, 5.5µl or 207µl thrombin (10U/ml, 269,300U/g) immediately before incubation at 37°C for 5min. Then, a two-fold concentrated Fn stock solution was subsequently mixed with cell suspension (containing tumor cells and/or PMNs) 1sec before experiments at 1:1 ratio to reach a desired Fg, thrombin, GPRP and cell concentrations.

4.2.4 Statistical analysis

Data were obtained from at least three independent experiments and expressed by the means. Statistical significance of difference between means was determined by using Student’s t-test or ANOVA. Tukey’s test was used for post hoc analysis for ANOVA. Probability values of P<0.05 were chosen as statistical significance.
4.2.5. Parallel Plate Flow Assay.

The adhesion experiments were performed in a parallel-plate flow chamber (Glycotech, Rockville, MD) mounted on the stage of a phase-contrast optical microscope (Diaphot 330, Nikon, Japan). The total magnification was set to 15x by using a 10x objective lens and 1.5 tube factor magnification. A CCD camera (pc0.1600; Cooke Corp, Romulus, MI) was used to record the experiments and it is connected to a PC. A syringe pump (Harvard Apparatus, South Natick, MA) was used to generate a steady flow field in the flow chamber. A Petri dish (35 mm) with a confluent EI cell monolayer (acting as a ligand-binding substrate) was attached to the flow chamber. All experiments were performed at 37°C.

Cell culture media was perfused into the chamber to allow monolayer to reach equilibrium for 1-2 minutes. Tumor cell suspension (10^6 cells/ml) was initially perfused at a higher flow rate (125/s) for approximately 40 sec to bring the cell into the field of view. After the initial perfusion time, the flow rate was decreased to a wall shear rate of 4.96/s (0.05 dyn/cm²) and kept constant for 3 minutes, which is the total time adhesive interactions counted. The field of view was 418 µm long and 400 µm wide and the frame rate was approximately 40 fps. Each experiment was repeated 3 times and analyzed offline.
4.3. Results.

4.3.1. Kinetics of sFn and ICAM-I binding.

ICAM-I is the most abundant receptor on tumor surface and share binding sites to sFg/sFn. We started analyzing the adhesive stops and plotting them into a histogram as a first step towards understanding the adhesive behavior. The logarithm of the number of the bound cells can give us a clue about the state of adhesiveness between the receptor and its respective ligand. At the first step of adhesion, the random rupture of attachments cause a linear trend of unbinding. However, in bond-strengthening process, the unbinding will show a logarithmic trend that will provide the dissociation constant of the given adhesive reaction (Vitte, 2004).

We have fit our first set of data to get an idea about the intrinsic property of ICAM-I~sFn~ICAM-I interaction. The first histogram (Fig 4.2) is the Lu1205 adhesive stops to EI cells. When we fit this data into a plot (Fig 4.3), we saw that it shows an exponential trend suggesting a kinetic relationship between sFn and ICAM-I molecule. Next step in this discovery will be to investigate the lifetime of the bonds by analyzing the videos with a image tracking software (Image Pro, Bethesda, MD). After this evaluation we can be able to get numbers for intrinsic $K_{off}$ rates for sFn and ICAM-I molecules under laminar flow conditions.
Figure 4.2. Histogram of the length of Lu1205 adhesions to EI monolayer in the presence of sFn. α,β3 on Lu1205 blocked to isolate the effect of sFn-ICAM-I interactions.

Figure 4.3. Approximate bond lifetime for sFn mediated ICAM-I-ICAM-I.
4.3.2. Kinetics of sFn and $\alpha_\beta_3$ binding.

When calculating the intrinsic $K_{off}$ values for $\alpha_\beta_3$-sFn~ICAM pairing in adhesion experiments we face to another important question about isolating the value of $\alpha_\beta_3$-sFn from sFn-ICAM-I. We analyzed the data with the same method and plotted respectively. The histogram (Fig 4.4) and the fitted graph suggest the preservation of the kinetic behavior with $\alpha_\beta_3$-sFn~ICAM as well.

Thus, the calculation of intrinsic $K_{off}$ from the lifetime plot will have both ICAM-I~sFn and $\alpha_\beta_3$~sFn interactions and it is essential to separate these two values from each other while getting $K_{off}$ for $\alpha_\beta_3$~sFn. This is the second necessary milestone needed to be accomplished in this study as a future research to accomplish.
Figure 4.4. Histogram of the length of Lu1205 adhesions to EI monolayer in the presence of sFn. ICAM-I on Lu1205 blocked to isolate the effect of sFn- \( \alpha_\text{v}\beta_3 \) interactions.

Figure 4.5. Approximate Bond Lifetime for sFn mediated \( \alpha_\text{v}\beta_3 \)-ICAM-I.
4.4 Discussions.

Fibrin mediated tumor cell endothelium interactions mainly take place between tumor cell $\alpha_v\beta_3$-ICAM-I and endothelial ICAM-I. The strength of the adhesion to endothelium is mainly dependent on the intrinsic kinetic rates of fibrin to $\alpha_v\beta_3$-ICAM-I receptors respectively. Our results show that blocking $\alpha_v\beta_3$ (Fig 4.2) does not have as much effect on tumor binding as the ICAM-I blocking case (Fig 4.4) for the number of stops for shorter time periods. ICAM-I $\sim$sFn$\sim$ICAM-I interactions supposed to be quantitatively greater than the $\alpha_v\beta_3$$\sim$sFn$\sim$ICAM-I interactions. The observed interactions would also be cause by the total number of receptors. It is shown in Table 2 that ICAM-I is the most abundant receptor on tumor cell membrane. If ICAM-I is the most abundant receptor, it is not surprising that there are more tumor cell endothelium interactions when $\alpha_v\beta_3$ is blocked. Considering this fact, it is important to take into consideration when calculating intrinsic dissociation rates for each receptors to sFn. There needs to be a closer look when distinguishing dissociation rates from $\alpha_v\beta_3$ to ICAM-I. Our results suggests ICAM-I is in favor of binding than $\alpha_v\beta_3$ but $K_{off}$ for $\alpha_v\beta_3$ might be bigger than $K_{off}$ for ICAM-I under same circumstances.

In conclusion, the first step towards estimating the intrinsic association and dissociation rates for melanoma cells retention on endothelium via sFn have been successfully completed and a crude idea about the kinetic interactions between two cell types predicted. The interactions should be studied towards getting a rate of change of the percentage of the remaining cells in order to estimate the apparent off-rates for the $\alpha_v\beta_3$ and ICAM-I.
4.5. Future Research Directions

The presence of sFn in tumor microenvironment can regulate the rates of the binding/unbinding interactions. It is very important to quantify the trend and get an apparent dissociation value for tumor cells interacting with the endothelium in the presence of fibrin. The data has an impact on designing possible drug targets for metastatic tumor cells with defined affinity values.

In this study, we have identified the kinetic behavior of previously shown $\alpha_v\beta_3$-sFn-ICAM-I and ICAM-I-sFn-ICAM-I interactions. We showed that sFn is an adhesive ligand for given receptors on tumor cells $\alpha_v\beta_3$ and ICAM-I and the trend shows a logarithmic slope suggesting the intrinsic $K_{\text{off}}$ value. As a next step the lifetime of the observed phenomenon need to be precisely quantified and the $K_{\text{off}}$ values for respective receptors would be calculated.
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Appendix:

Thawing Cells (Vials are stored in liquid nitrogen)

When removing a vial from liquid nitrogen, twist the cap a little to release pressure in the vial (Sometimes, the top pops open due to increase in internal pressure). Prepare 4ml medium (90% DMEM+10%FBS) and warm media to 37 C in a 100mm Petri dish. 

**Remember: Freeze slowly, Thaw quickly.** Defrost the vial of cells in a water bath. Let a small chunk of ice remain in the vial, this maintains temperature of the cells and thus prevents DMSO in the freezing medium from permeabilizing the cell membrane. Pipette cells into warmed medium in the dish and label with date and passage number. It will contain total 10ml (9ml medium+1ml cells from the frozen vial). Place the dish in the incubator.After ~6-10hrs replace the medium in the flask.

Passing Cells (Cells should be passed when they are ~70-90% confluent)

Prepare 30ml medium for passing cells (place in a centrifuge tube & warm in water bath). Warm Trypsin (1ml per dish) & DPBS without Ca & Mg (~15ml per flask). Wash cells to be passed with ~5mL of pre-warmed DPBS, 2 times. Place 1 mL of trypsin in the dish and monitor under microscope till the cells begin to round-up. This will take ~1-5 mins. Then aspirate most of the trypsin (leave ~0.2ml in the dish). Gently tap the dish to detach cells. Examine under the microscope. If the cells have detached then add 10ml pre-warmed medium prepared in Step 1 else, add more tryspin & wait for ~1min for the cells to round-up & detach. Gently wash and agitate the medium in the dish to detach & break clumps of cells.
Add 10ml of prepared media for each new dish and add 10ml to the old dish with the cells, suspending them. Place 1mL of passing media into each dish depending on desired seeding density (~3600 cells/cm²). Label plate with name of cells, date, passage number, and your name. Place in incubator with 90-95% humidity, 5% CO₂, 37°C temperature. The cells will be confluent in 2 to 3 days depending on seeding density. Check cells daily and replace medium as necessary (every other day for primary cultures).

**Cryopreserving Cells**

We will need 4 confluent dishes. Warm 10ml passing medium, Ca & Mg free DPBS & trypsin. Prepare freezing media (10mL) 50% FBS / 5%DMSO / 45% cell medium (MCDB-131). Maintain at 4C. Prepare 10 cryopreserving vials, keep them on ice. Label each vial with cell type, passage number, date. Wash each dish three times with DPBS. Add 1ml trypsin to each dish & monitor cells under microscope. When cells start rounding-up, aspirate most of the trypsin. Add some part of the 10ml washing medium to each flask. Tap gently to detach cells. Gently aspirate & release the medium a few times to detach all the cells & break clumps. Collect medium from all dishes into a centrifuge tube. Centrifuge cells suspended in passing medium at 1200 rpm for 5 minutes. Aspirate off supernatant and resuspend in Freezing medium. Aliquot freezing medium from step 9 into 1 ml vials, place each vial on ice immediately. Put vials into freezing box and place into –80 freezer (temperature of cells should decrease 1°C/min). After a few days, thaw 1 ampule to check cell viability. For long-term storage, cells should be placed in liquid nitrogen.
Informed Consent Form: The Pennsylvania State University
Title: Molecular Dynamics in Neutrophil-facilitated Tumor Cell Adhesion and Extravasation
Study Approval Number: IRB#31120
Principal Investigator: Cheng Dong; 233 Hallowell Bldg., University Park, PA 16802; cxd23@psu.edu; (814) 865-8091
Other Investigators: Pu Zhang, Changliang Fu, Tugba Ozhemir, Yi Fu

Participant Name: ____________________________________________________________

Background for the Study
Cancer is the second leading cause of death in the United States. The ability of cancer to spread is one reason why cancer is so deadly and treatment difficult. If the cancer can be contained to one tumor, the survival rate is much greater than for patients with cancer that has spread from one organ to another organ. Successful spread of cancer requires a series of complex steps. These steps can be summarized into four main steps: 1) invasion 2) transport from one area of the body to another 3) sticking of cancer cells to blood vessels and 4) crossing of cancer through the blood vessel into neighboring organs. Inhibition of any of the steps in these steps will prevent or slow the spread of cancer. We propose to study how cancer cells stick to blood vessels and study the process by which cancer cells move through blood vessels into organs. This study is being conducted for research purposes only.

Methods and associated risks
A 30 minute visit will be required of each subject where each blood draw session could take up to 45 minutes. At this visit, a 30-100 ml (100ml is less than 7 tablespoons) sample of blood will be drawn for the purpose of collecting white blood cells from the whole blood. As a participant, the amount of blood drawn will not exceed 550 ml (550 ml is a little over 37 tablespoons or a little over 1 pint) in an 8 week period and collection may not occur more frequently than 2 times per week. All unused portions of the blood will be disposed. The blood draw can result in local discomfort, fainting, inadvertent puncture of an artery and a small bruise at the site of the puncture. Clots and infection are also potential risks. Having a trained, proficient technician perform the procedure using sterile techniques on a participant who is lying down will minimize all risks.

As a general health screen, you must be able to answer NO to the following questions each day that you donate a blood sample.
• Are you under 18 years of age?
• Do you weigh less than 110 lbs?
• If female, are you pregnant?
• Are you anemic (a disease where you have low blood iron) or have a history of low blood iron?
• Are you taking prescription antibiotics?
• Are you taking allergy or cold medication?
• Do you have or do you have a history of HIV or Hepatitis infection?
• Have you had mononucleosis (a disease where you have fever, sore throat, and fatigue) in the past 6 months?
The initial blood draw will include 1 teaspoon of blood for a lab test called a complete blood count with differential cell count (CBC with diff). Complete blood counts are taken because the CBC can reveal anemia (low red blood cell count), leukopenia (low white cell count) and thrombocytopenia (low platelet count) which may indicate an underlying condition or illness. In the event that abnormal blood count test results are obtained, you will be apprised of the results and advised to contact your medical provider for follow-up.

Blood samples collected for this study will only be used for research purposes. There is the possibility that your body’s genetic material will be extracted from your white blood cells, in the case that it is, the sample will not have personal identifiers on it, will not be stored for longer than 6 months and will be shared only with people associated with this project. Experiments will not test for any disease and results from your specific blood sample will not be available, so results will not be shared with you or your insurance company.

Confidentiality of records
All data will be kept in coded files and locked in 307 Hallowell. All data will not be available or divulged to anyone outside of the experimental research team except those who “need to know” for scientific purposes involved in carrying out this study. Your blood sample will never be labeled with your name or an identifying label. All CBC information with the patient’s information if any is kept with GCRC nurses in secure files and no part of this CBC information will be divulged to investigators or anyone else. The following may review and copy records related to this research: The Pennsylvania State University’s Office for Research Protections and Institutional Review Board, and the Office for Human Research Protections in the Department of Health and Human Services.

Potential Benefits
By voluntarily participating in this study, you will be contributing to the general body of knowledge about the metastasis and progression of cancer, thereby providing a public service. If you choose, you may receive a copy of the results from your CBC with differential screening test.

Compensation
For your time, you will be paid $15 per blood draw.

Voluntary Participation and Right to Ask Questions
______________________ (participant’s full name), having the full capacity to consent does hereby volunteer to participate in a research study entitled “Molecular dynamics in leukocyte assisted melanoma extravasation” under the primary direction of Cheng Dong, Ph.D, Professor of Bioengineering. The implications of the participant’s voluntary participation, the nature, duration and purpose, the method and means by which the study is to be conducted, and any inconveniences or hazards which may be reasonably expected have been explained to the participant and are set forth on the prior page of this agreement.

The participant has been given the opportunity to read and keep a copy of this informed consent document and understand the participant will be compensated $15.00 for each blood sample. Please contact Cheng Dong at (814)865-8091 with questions, complaints or concerns about this research. You can also call this number if you feel this study has harmed you. If you have any questions, concerns, problems about your rights as a research participant or would like to offer input, please contact The Pennsylvania State University’s Office for Research Protections (ORP) at (814) 865-1775. The ORP cannot answer questions about research procedures. Questions about research procedures can be answered by the research team.

In participating in the study, there are no foreseeable discomforts or risks to the participant. In the unlikely event you become injured as a result of your participation in this study, medical care is available but neither financial compensation nor free medical treatment is provided. By signing this document, you are not waiving any rights that you have against The Pennsylvania State University for injury resulting from negligence of the University or its investigators.
The participant’s decision to be in this research is voluntary. The participant can stop at any time. The participant does not have to answer any questions they do not want to answer. Refusal to take part in or withdrawing from this study will involve no penalty or loss of benefits they would receive otherwise.

The participant is free to withdraw consent and terminate their participation at any time without prejudice.

Participant Signature: ____________________________
Date: ___________________

I have defined and fully explained the investigation to the above participant.

Investigator Signature: ____________________________
Date: __________________

Participant Signature: ____________________________
Date: ___________________

I have defined and fully explained the investigation to the above participant.