Fibrin serves as a divalent ligand that regulates
neutrophil-mediated melanoma cells adhesion to
endothelium under shear conditions

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Running title: Soluble fibrin enhances melanoma adhesion under flow conditions

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ABSTRACT

Elevated soluble fibrin (sFn) levels are characteristic of melanoma hematogeneous dissemination, where tumor cells interact intimately with host cells. Melanoma adhesion to the blood vessel wall is promoted by immune cell arrests and tumor-derived thrombin, a serine protease that converts soluble fibrinogen (sFg) into sFn. However, the molecular requirement for sFn-mediated melanoma-polymorphonuclear neutrophils (PMNs) and melanoma-endothelial interactions under physiological flow conditions remain elusive. To understand this process, we studied the relative binding capacities of sFg and sFn receptors e.g., αvβ3 integrin and intercellular adhesion molecule 1 (ICAM-1) expressed on melanoma cells, ICAM-1 on endothelial cells (EC), and CD11b/CD18 (Mac-1) on PMNs. Using a parallel-plate flow chamber, highly metastatic melanoma cells (1205Lu and A375M) and human PMNs were perfused over an EC monolayer expressing ICAM-1 in the presence of sFg or sFn. It was found that both the frequency and lifetime of direct melanoma adhesion or PMN-facilitated melanoma adhesion to the EC in a shear flow were increased by the presence of sFn in a concentration-dependent manner. In addition, sFn fragment D and plasmin-treated sFn failed to increase melanoma adhesion, implying that sFn-bridged cell adhesion requires dimer-mediated receptor-receptor crosslinking. Finally, analysis of the respective kinetics of sFn binding to Mac-1, ICAM-1 and αvβ3 by single-bond cell tethering assays suggested that ICAM-1 and αvβ3 are responsible for initial capture and firm adhesion of melanoma cells. These results provide evidence that sFn enhances melanoma adhesion directly to ICAM-1 on the EC, while prolonged shear-resistant melanoma adhesion requires interactions with PMNs.

Key words: shear rate; PMN; melanoma cells; EC; fibrin; ICAM-1; αvβ3
INTRODUCTION

Melanoma metastasis consists of highly regulated molecular events, including tumor detachment from the primary lesion, translocation within the blood circulation, successful adhesion to and extravasation from the walls of capillary vessels in target tissues (13). During their translocation to the lung capillaries, melanoma cells are subject to mechanical shear forces and interact with plasma proteins and immune cells that might regulate tumor cell (TC) adhesion and survival (31). Therefore, elucidating the interplay between these blood components and melanoma cells within the intravascular tumor microenvironment is critical for understanding the mechanism of metastasis.

Correlation between tumor metastasis and activation of blood coagulation has been described (65, 77). Specifically, several studies indicated that soluble fibrinogen (sFg) and fibrin (sFn) act as a rate-limiting step in primary capture of circulating melanoma cells (7, 12, 19, 28, 54). The pro-coagulant potential ascribed to melanoma cells has been linked to a transmembrane protein tissue factor (TF). Metastatic melanoma cells were reported to express 1000-fold higher levels of TF on their membranes than non-metastatic cells (44). The membrane-bound TF triggers coagulation by facilitating thrombin generation (66). Thrombin is a serine protease responsible for many homeostatic functions, including conversion of sFg into sFn and activation of various intercellular signaling events in circulating blood cells via protease activated receptor-1 (PAR-1) (17). Under certain circumstances, thrombin exposure may result in cellular inflammatory responses, such as altered intercellular adhesion molecule-1 (ICAM-1) expression on vascular endothelial cells (ECs) and activation of polymorphonuclear neutrophils (PMNs) (75, 84). The aim of the present study was to elucidate the mechanisms of melanoma cell adhesion within the vascular tumor microenvironment, focusing on the role of thrombin as a factor affecting fibrin formation and melanoma adhesion. By altering their environmental variables, melanoma cells have developed several mechanisms for successful adhesion to the ECs under flow conditions. For example, melanoma cells are known to express high levels of ICAM-1, which is an immunoglobulin superfamily molecule mediating leukocyte firm adhesion to activated ECs (50, 74). Additionally, ICAM-1 has been
associated with epithelial carcinogenesis (57). Inhibition of ICAM-1 on tumor cells of different tissue
origins, either by small interfering RNA (siRNA) or blocking monoclonal antibody, led to a strong
suppression of tumor invasion (13). Besides ICAM-1, melanoma cells express a variety of integrin
molecules, including fibrin(ogen)-receptor integrin \( \alpha_v\beta_3 \) (60). Expression of \( \alpha_v\beta_3 \) conferred the potential
for angiogenesis and metastasis on melanoma (3, 58, 81). An increased level of sFg and sFn around
circulating melanoma cells promoted melanoma adhesion, possibly due to the fact that both sFg and sFn
are ligands for cells expressing ICAM-1 and \( \alpha_v\beta_3 \) (20). In addition, sFn has been shown to enhance
melanoma cell adhesion to platelets under flow conditions (7).

PMNs have recently been suggested to play a role in tumor-host interactions. It has been shown that
patients with bronchioloalveolar adenocarcinoma have poor clinical outcomes when PMNs infiltrate
tumor tissues (80). The role of PMNs in promoting melanoma metastasis has been supported by in vivo
investigations (27). In several previous studies, PMNs were suggested to promote melanoma adhesion to
the endothelium via ICAM-1 interactions (35, 36, 71, 72). In addition, fibrin(ogen) could bind to
CD11b/CD18 (Mac-1) on PMNs in flow (21, 42), thereby facilitating PMN adhesion to the ECs. In light
of these previous studies, it remains highly possible that the coagulation process may affect both
melanoma direct adhesion to the ECs and PMN-melanoma interactions. However, the mechanism for
cooperative regulation of melanoma cell adhesion by immune responses and coagulation events is
currently unknown.

A recent study indicated that sFn potentiated melanoma-PMN heterotypic aggregation (83). In the
current study, we hypothesize that sFn produced in a tumor microenvironment supports direct melanoma
interactions with the endothelium and enhances PMN-facilitated melanoma adhesion within the
circulation. In fact, we characterized the dynamics of adhesion using a flow chamber and showed that
melanoma cell adhesion to PMNs through sFn was not due to nonspecific capture, but rather a
receptor-mediated interaction crosslinking ICAM-1 or \( \alpha_v\beta_3 \) on melanoma cells to the ICAM-1 on the
endothelium. Here, sFn was made by reacting sFg and thrombin in the presence of anticoagulant,
Gly-Pro-Gly-Gly (GPRP). GPRP could bind to fibrinogen D domain, inhibiting transglutaminase
crosslinking by changing the glutamine residues in the α and γ chains of fibrinogen (1, 69). It has been shown that GPRP can prolong the fibrinopeptide B release without affecting fibrinopeptide A release kinetics (47). In order to demonstrate the divalent binding mechanism, sFn was degraded to fragment D by plasmin-limited digestion. Fragment D primarily consists of an intact COOH-terminal stretch of the γ chain containing the ICAM-1, αvβ3 and Mac-1 binding regions. In the presence of fragment D and plasmin-digested sFn, melanoma adhesion was significantly reduced, suggesting that melanoma-EC adhesion requires a divalent binding mechanism.

We further investigated the kinetics of these crosslinking processes and the relative importance of each individual receptor-ligand interaction for successful melanoma adhesion. The tethering experiments showed that melanoma cells initiated interactions with the ECs and PMNs in the presence of sFn. However, the crosslinked sFn bonds formed between Mac-1 on PMNs, and ICAM-1 (or αvβ3) on melanoma cells were significantly stronger than the crosslinked sFn bonds formed between endothelial ICAM-1 and melanoma ICAM-1 (or αvβ3). These data demonstrate that although sFn increases the frequency of bond formations between melanoma and ECs, PMNs can stabilize these bonds and prolong bond lifetime.

MATERIALS AND METHODS

**Cell preparations and Reagents.** A375M and 1205Lu metastatic melanoma cells (kindly provided by Dr. Gavin Robertson, Penn State Hershey Medical Center, PA) were grown in DMEM/F12 (Dulbecco’s Modified Eagle Medium Nutrient Mixture F12) supplemented with 10% fetal bovine serum (FBS). Adhesion molecule expressions of selected cells were detected by flow cytometry described elsewhere (34) (Table 1). For flow experiments, a confluent monolayer of melanoma cells was detached by using 0.05% trypsin/EDTA and the detached cells were washed twice in a fresh culture medium. The cells were then suspended in fresh media and allowed to recover for 1 hr while being rocked at a rate of 8 rpm at 37°C. For receptor blocking experiments, A375M and 1205Lu cells were pre-treated with functional
blocking antibodies (anti-αvβ3 or anti-hICAM-1; 5 µg/ml) prior to perfusing the cells into the flow chamber.

PMN preparation was described in detail elsewhere (37). Briefly, following The Pennsylvania State University Institutional Review Board (IRB)-approved protocols (no. 19311), fresh human blood was collected from healthy adults by venipuncture. PMNs were isolated using a Ficoll-Hypaque (Histopaque, Sigma) density gradient method as described by the manufacturer. Isolated PMNs were maintained at 4°C in Dulbecco’s phosphate-buffered saline (DPBS) containing 0.1% human serum albumin (HSA) for up to a maximum of 4 hrs prior to conducting an experiment.

Genetically modified fibroblast L-cells that express stable levels of human E-selectin and ICAM-1 (generously provided by Dr. Scott Simon, UC Davis, CA) were used in the current study as a model of the endothelial monolayer substrate (referred as EC). Transfected L-cells express ICAM-1 at a level comparable to IL-1β stimulated human umbilical vein endothelial cells (24).

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 Mac-1 (anti-CD11b) and Cell Tracker Green were purchased from Invitrogen Corporation (Carlsbad, CA). N-formyl-methionyl-leucyl-phenylalanine (fMLP), sFg (Fraction I, type I: from human plasma), GPRP (Gly-Pro-Arg-Pro amide), aprotinin and BSA (bovine serum albumin) were purchased from Sigma (St Louis, MO). Bovine thrombin (269,300 U/g) was purchased from MP Biomedicals (Solon, Ohio). Fragment D was purchased from Haematologic Technologies Inc. (Essex Junction, VT).

1 ml of fresh soluble sFn (per 1 × 10⁶ cells) was prepared by incubating 120 µl sFg (25 mg/ml) and 84 µl GPRP (24 mM) with 200 µl of thrombin (from 10 U/ml stock) at 37 °C for 5 min prior to perfusion experiments. This method prevents the polymerization of sFn molecules upon thrombin cleavage (10). A two-fold concentrated sFn stock solution was mixed with cell suspension (containing melanoma cells and/or PMNs) immediately before perfusion at 1:1 ratio to obtain desired mixture conditions. To obtain other proteins in the mixture, fibrin polymers were formed by reacting 1.5 mg/ml sFg and 2 U/ml thrombin at 37
ºC for 30 min. Fibrin polymers were removed by swirling with a pipette tip and filtration with 0.2 μm pore filter.

Preparation of fibrin fragments was conducted with limited plasmin digestion of the synthesized fibrin in 150 mM NaCl and 20 mM HEPES at PH 7.4 (19). 0.02 U of plasmin per mg fibrin(ogen) in the presence of 1 mM CaCl₂ at 37 ºC for 2 hr was used to cleave sFn. The reaction was stopped by adding 500 KIU aprotinin/U of plasmin.

Flow Assays. The adhesive interactions between 1205Lu cells, PMNs (stimulated with 1 μM fMLP for 1 min before perfusion to the chamber), and EC via sFg or sFn were quantified using a parallel-plate flow chamber system (Glycotech, Rockville, MD). Cell suspensions (1205Lu:PMN at 1:1 ratio of 1 × 10⁶ cells/ml each) were mixed with or without plasma proteins (sFg, sFn or fragments) and perfused at a desired hydrodynamic shear rate into the flow chamber using a syringe pump (Harvard Apparatus, South Natick, MA). After being settled for a period of time at a shear rate of 10 sec⁻¹, cells were subjected to an experimental shear rate of 62.5 and 200 sec⁻¹, respectively. Phase contrast images of cells near the EC surface were captured and recorded for 3 min at a frame rate of 30 fps and analyzed offline.

Quantification of cell-cell and cell-substrate interactions. Interactions of circulating cells and EC were classified into three categories: (1) melanoma cell direct arrest on the EC; (2) transient PMN tethering on the EC; and (3) PMN-mediated melanoma cell arrest on the EC. Time scales of these interactions in the presence or absence of sFg or sFn were measured as a means to study the crosslinking adhesion mechanisms. In order to characterize the dynamics of these interactions, we categorized the durations of interactions (t) into short (1 sec < t < 3 sec), intermediate (3 sec < t < 5 sec), and long (t > 5 sec) term arrests. Although the binding durations of melanoma-EC interactions could be detectable as short as several msec, only the longer tethers (> 1 sec), which were anchored by multi-bonds and contributed to the final melanoma adhesion, were further analyzed. Then, within each time category, the frequency of adhesion of melanoma cells or PMNs on the EC per min per mm² was quantified. PMN tethering frequency was
normalized and expressed by number of tethered PMNs per unit area per unit time (48, 67, 72). In addition, the normalized frequency of melanoma cell binding to the EC monolayer was defined as the number of cell arrest events per unit area and unit time. For PMN mediated melanoma interaction with the EC, a term of “Adhesion Efficiency” was defined as follows:

\[
\text{Melanoma Adhesion Efficiency} = \frac{\text{Number of melanoma cells arrested on the EC as a result of collision to tethered PMNs in a given duration}}{\text{Total number of melanoma-PMN collisions}}
\]

The numeration of cell adhesion in each case was confirmed by a second investigator who was not informed of the objective of the case in order to ensure unbiased measurement.

Tethering experiments for single bond dissociation rate measurement. To reveal the mechanical properties of these adhesions, tethering experiments were performed to obtain the force regulated bond dissociation rates by measuring lifetimes of the transient tethers at shear rates of 62.5 and 200 sec\(^{-1}\). The A375M cell line was employed for tethering experiments since A375M cells have lower expressions in ICAM-1 and \(\alpha_v\beta_3\) compared with the 1205Lu cells (Table 1). A375M cells were stained with Cell-Tracker Green and resuspended in fibrin solution immediately before the perfusion experiments to minimize pre-binding of cell receptors with sFn.. The cell suspension was then perfused over the EC in the flow chamber at shear rates of 62.5 and 200 sec\(^{-1}\), respectively. The images of tethering experiments were captured with an exposure time of 30-50 msecs using fluorescence microscopy and converted to Audio Video Interleave (avi) files for further analysis by Image-Pro Plus (Media Cybernetics, Silver Spring, MD). A total of 100-150 events were analyzed for each case to estimate the apparent bond dissociation rate (\(k_{off}\)) by fitting the lifetime to \(P=1-\exp(-k_{off}t)\), where \(P\) is the probability of bond formation (2). Only short-term arrests (< 2 sec) were used to calculate the dissociation rates (2, 14, 78) as \(k_{off}\) was mainly determined by short-term single bond arrests.

To verify a sFn-crosslinking reaction for bond dissociation rate constants, three experiments were performed with the following aims: (1) To measure the \(k_{off}\) for \((\text{ICAM-1})_{TC-(sFn)-(ICAM-1)}_{EC}\) bond,
α,β3-blocked A375M cells were perfused over the EC; (2) To obtain the $k_{off}$ for
$(\alpha_1\beta_3)_\text{TC}-(\text{sFn})-(\text{ICAM-1})_\text{EC}$ bond, ICAM-1-blocked A375M cells were perfused over the EC; (3) To get
the $k_{off}$ for (Mac-1)-(sFn) bonds, fMLP-activated PMNs were perfused over a sFn coated surface (2.5
mg/ml sFg was immobilized on the substrate of a flow chamber before being catalyzed by 2 U/ml
thrombin for 2 hr to generate Fn).

**Estimation of bond affinity from a probability model.** In order to understand the bond kinetics from both
association and dissociation rates, we further conducted kinetic simulations based on a well-developed
probabilistic theory (15):

$$dp_n(t)/dt = k_{on}p_{n-1} - (k_{on} + nk_{off})p_n(t) + (n + 1)k_{off}^{(n+1)}p_{n+1}(t).$$

Here $p_n(t)$ is the probability of having $n$ bonds at time $t$ and $k_{on}$ is the apparent association rate. An
apparent binding affinity was determined by $K_a = k_{off}/k_{on}$. For cell detachment, Eqn 1 was modified by
setting the transient probability from 0 to 1 bond as zero. The initial condition is that all arrested cells are
most likely linked by only one bond initially (i.e. $p_1 = 1$ and $p_n = 0$ when $n = 0, 2, \ldots$). Since most of the
cells were detached within 5 min, we assumed the remaining arrested cells are linked only by few bonds,
and the hydrodynamic force acting on each cell is shared by these bonds. It is important to note that those
formed bonds break sequentially following a well-established probabilistic theory (15, 16, 23). A
Rung-Kutta numerical scheme and a Levenberg-Marquart method (22, 37) were used to fit the above
model to the detachment data with the $k_{off}$ measured by tethering experiments. The probability of short,
intermediate, and long arrests ($p_{\text{short}}$, $p_{\text{inter}}$ and $p_{\text{long}}$) were calculated by Eqn 2 and compared with the
experimental results.

$$
\begin{align*}
    p_{\text{short}} &= \left[p_n(t_2) - p_n(t_1)\right]/\left[1 - p_n(t_1)\right], \\
    p_{\text{inter}} &= \left[p_n(t_3) - p_n(t_2)\right]/\left[1 - p_n(t_1)\right], \\
    p_{\text{long}} &= \left[1 - p_n(t_3)\right]/\left[1 - p_n(t_1)\right],
\end{align*}
$$

where $t_1 = 1$ sec, $t_2 = 3$ sec and $t_3 = 5$ sec according to the experimental procedure.
**Statistical analysis.** All data were obtained from at least three independent experiments and expressed by mean ± standard error of the mean (SEM). Statistical significance was determined using Student’s t-test or ANOVA. Tukey’s test was used in post hoc analysis for ANOVA. Probability value of $P < 0.05$ was considered to be statistically significant. For tethering experiments, the 95% confidence intervals for regression fitting of unbinding curves were plotted using Sigmaplot (Systat Software, San Jose, CA).

**RESULTS**

**Soluble fibrin supports melanoma-endothelium adhesion.** To determine whether sFn mediates melanoma adhesion to ECs, human metastatic 1205Lu melanoma cells were perfused over a confluent EC monolayer expressing ICAM-1. Direct cell adhesion to EC was analyzed and categorized into short, intermediate and long-term arrests, as described in Materials and Methods to reflect different phases of cell adhesion. Fig. 1A-C compares the effects of sFg and sFn on melanoma adhesion with respect to the control conditions (without sFg or sFn) at different shear rates (62.5 and 200 sec$^{-1}$).

1205Lu alone interacted minimally with the EC substrate as they only had 60 short, 17 intermediate and 10 long-term arrests within 1 min. Adding 1.5 mg/ml sFg increased the short-term arrests by 50% (Fig. 1A “short”), intermediate arrests by 160% (Fig. 1A “intermediate”) and long-term arrests of firm cell adhesion by 300% (Fig. 1A “long”) at a shear rate of 62.5 sec$^{-1}$. Compared to sFg alone, addition of sFn further increased melanoma arrests at all three time intervals To determine the role of ICAM-1 on EC in facilitating melanoma adhesion via sFn, ICAM-1 was functionally blocked on the EC by 5 μg/ml mAb before being used as a substrate for cell adhesion. ICAM-1-blocked EC were unable to mediate sFn-bound 1205Lu cell adhesion while ICAM-1-expressing EC maintained this ability(Fig. 1B). This suggests that EC ICAM-1 is the primary receptor for fibrin(ogen)-mediated melanoma binding to the EC under flow conditions.

Increasing shear rate from 62.5 to 200 sec$^{-1}$ altered the relative contributions of sFg and sFn to the melanoma arrests (Fig. 1C). At 200 sec$^{-1}$, sFn resulted in a marked increase in intermediate and long-term...
adhesion frequencies. For intermediate-term adhesion, sFn elevated 1205Lu adhesion by two-fold, while sFg did not significantly change melanoma cell adhesion (Fig. 1C “intermediate”). sFn also increased the frequency of long-term adhesion by four-fold compared with sFg (Fig. 1C “long”). Results from Fig. 1A-C provide strong evidence for the claim that sFn supports both the initial short-term arrest and long-term firm adhesion at both low and high shear rates.

The relative roles of ICAM-1 and αvβ3 on melanoma cell sFn crosslinked adhesion to ICAM-1 on the EC were determined by functionally blocking the respective receptors on the melanoma cells before perfusion at 62.5 and 200 sec⁻¹ in the presence of sFn (Fig. 1D and E). Short-term melanoma-EC interactions were shown to be mainly mediated by ICAM-1 on the melanoma cell, since blocking ICAM-1 reduced sFn-mediated adhesions by more than 50%, while blocking αvβ3 had less significant effects. Blocking ICAM-1 or αvβ3 prevented intermediate and long-term melanoma adhesions, suggesting ICAM-1 and αvβ3 expressed on melanoma cells are required for longer-term sFn-mediated adhesions to the EC under shear conditions. Other receptors did not seem to play a role in sFn-mediated 1205Lu attachment, since when ICAM-1 and αvβ3 were blocked simultaneously, there were no cell arrests at both 62.5 and 200 sec⁻¹ (data not shown).

sFg is a glycoprotein that comprises two sets of three-polypeptide chains, Aα, Bβ, and γ, linked together by 29 disulfide bonds. This dimerization brings together two identical binding sequences and makes fibrinogen a divalent ligand. To test whether this divalent ligand-mediated crosslink binding would be important for melanoma direct adhesion, sFn was cleaved by plasmin to fragment E and D. Fragment D consists of one set of C-terminals of Aα, Bβ, and γ chain, involving two αvβ3, two Mac-1 and one ICAM-1 binding sites. When sFn was replaced with fragment D, the intermediate and long-term melanoma arrests were significantly reduced (Fig. 2A). Likewise, plasmin-treated sFn failed to mediate melanoma adhesion. Other proteins in the sFn solution (which was readily obtained by first allowing fibrin to polymerize, removing fibrin polymers with filtration, and then adding GPRP) were also incapable of inducing melanoma adhesion, suggesting intact sFn was the only mediator to facilitate melanoma direct adhesion to the EC. Low thrombin levels partially converted sFg to sFn. In the presence of 0.053 U/ml
thrombin-generated sFn, melanoma adhesion frequencies were significantly reduced for all arrest durations (Fig. 2B). Compared with sFn made by 1.5 mg/ml sFg, sFn from 15 μg/ml sFg was incapable of mediating cell firm adhesion.

**Soluble fibrin enhances PMN tethering to endothelial ICAM-1.** Activated PMNs were shown to facilitate the melanoma adhesion to the endothelium via adhesive interactions between β2 integrin (LFA-1 and Mac-1) on PMN and ICAM-1 on melanoma cells (36, 71). PMNs exhibited enhanced rolling, tethering and adhesion to the EC. fMLP stimulation has been shown to upregulate Mac-1 expression level by nine-fold (70). Therefore, in order to exclude the potential activation of PMNs by thrombin, PMNs were pre-stimulated by fMLP before being perfused into the flow chamber. To probe possible roles of sFn crosslinking in PMN adhesion to ECs, PMN tethering to the ICAM-1 was quantified. Results from Fig. 3 show that sFn increases PMN adhesion by two-fold at a shear rate of 62.5 sec⁻¹. Increasing shear rate significantly decreases the PMN tethering frequency. Our results are consistent with findings from previously published work (33), suggesting that sFn plays a more important role than sFg in mediating activated PMN firm adhesion to the EC under high shear conditions. When ICAM-1 was blocked, the lifetime of PMN arrests on the EC decreased and cells displayed fast rolling velocities (data not shown).

**Soluble fibrin regulates PMN-facilitated melanoma adhesion to ICAM-1.** To evaluate the role of sFn on PMN-mediated melanoma cell adhesion to the endothelium, the ability of 1205Lu cells to interact with adherent PMNs on the EC was quantified, in terms of “Adhesion Efficiency” (defined in the Materials and Methods). Adhesion Efficiency was used here because not all melanoma-PMN collisions could successfully result in aggregate formations on the EC under flow conditions. In order to compare the cases of PMN-facilitated melanoma adhesion and direct melanoma adhesion to the EC, the time durations that melanoma-PMN aggregates stayed in a close proximity of the EC were categorized into short, intermediate and long-term arrests. We showed that sFn significantly enhanced the rolling, tethering and arrest of PMNs on the EC monolayer (Fig. 3). This increased the probabilities of PMN-1205Lu cell collisions, which
further facilitated 1205Lu adhesion to the EC under flow conditions. From our observations, the process of PMN-facilitated melanoma adhesion follows three steps: (1) a melanoma cell from upstream collides with a tethered PMN; (2) the melanoma cell transiently adheres to a tethered PMN; and (3) the melanoma cell arrests on the EC by forming an aggregate with a tethered PMN.

sFg significantly increased short-term PMN-mediated 1205Lu Adhesion Efficiency at 62.5 sec\(^{-1}\) (0.34±0.020 compared with 0.20±0.015 from the control) and 200 sec\(^{-1}\) (0.25±0.015 compared with 0.13±0.016 from the control) (Fig. 4A and B). However, sFg did not significantly affect intermediate and long-term Adhesion Efficiency of melanoma cells. Compared with sFg under both shear conditions, sFn increased the short-term Adhesion Efficiency by more than two-fold (Fig. 4A and B). In contrast to direct melanoma adhesion (Fig. 1A), sFn did not significantly increase long-term PMN-mediated melanoma adhesion at a relatively low shear rate of 62.5 sec\(^{-1}\), but promoted sustained adhesion at a high shear rate of 200 sec\(^{-1}\) (Fig. 4A and B). It was noted that even when ICAM-1 on EC was inhibited, intermediate and long-term melanoma Adhesion Efficiencies were not significantly affected, although the absolute frequency of melanoma tethering via PMN was reduced (Fig. 4C). This might be due to the fact that PMN-mediated melanoma Adhesion Efficiency was dependent on sFn crosslinking between PMNs and melanoma cells, which was not affected by the ICAM-1 blocking on the EC.

To assess the role of sFn receptors in melanoma Adhesion Efficiency, we functionally blocked ICAM-1 and \(\alpha_v\beta_3\) respectively on the melanoma cells and quantified the Adhesion Efficiency at shear rates of 62.5 and 200 sec\(^{-1}\), respectively. Results from Fig. 4D and E showed that the short-term PMN-melanoma interactions were apparently initiated by an engagement of ICAM-1 on melanoma (TC) and Mac-1 on PMN crosslinked by sFn, while \((\alpha_v\beta_3)_{TC}-(sFn)-(Mac-1)_{PMN}\) bonds could prolong the lifetime of PMN-enhanced melanoma adhesion.

To verify the divalency of sFn-mediated binding, sFn (0.053 U/ml thrombin) in the binding solution used in the above adhesion assays was replaced by 1.5 mg/ml fragment D from sFn or plasmin-digested sFn. Binding for all arrest durations was prevented, suggesting that sFn-mediated PMN-melanoma aggregation follows a divalent crosslinking mechanism (Fig. 5A). This binding was specific, since other proteins did not
facilitate any binding after fibrin polymers were removed. sFn made from a low concentration of thrombin (0.053 U/ml) did not significantly increase the magnitude of PMN-mediated adhesion compared with 1.5 mg/ml sFg alone without thrombin (Fig. 5B). In sharp contrast to higher sFn concentration, lower sFn concentration effectively reduced melanoma Adhesion Efficiency (Figs. 4A vs 5B). This may imply that low concentrations of sFn weakened the binding between ICAM-1 and Mac-1 and/or between ICAM-1 and LFA-1, thereby inhibiting PMN-mediated melanoma adhesion.

**Bond apparent dissociation rates and affinities reflect relative contributions of ICAM-1 and αvβ3.**

Apparent dissociation rate $k_{off}$ for sFn receptors ICAM-1, αvβ3, and Mac-1 were determined by single-bond tethering assay (26, 59). As is shown in Fig. 6A, the trajectories and locations of fluorescently labeled cells in each frame could be conveniently tracked by Image-Pro Plus. The algorithm in the software correlated the cell positions in a series of frames and cell accumulative distance was plotted as a function of time (Fig. 6B). The plateau in the curve represents the lifetime of the bond (indicated as $dt$). Adhesion of fMLP-stimulated PMNs on immobilized sFn had a $k_{off}$ of 1.60 sec$^{-1}$ at a shear rate of 62.5 sec$^{-1}$ and 2.83 sec$^{-1}$ at 200 sec$^{-1}$ (Fig. 6C). These values fall into a range of the force-free dissociation rate of PSGL-1 and selectin interactions (1–10 sec$^{-1}$) (41). Since Mac-1 is the only known receptor for sFn on PMNs, these $k_{off}$ values for (Mac-1)-(sFn) bonds might indicate that shear force had very little effect on this type of bond, since a 95% confidence level of the values measured at 62.5 and 200 sec$^{-1}$ overlapped (Fig. 6C).

A375M melanoma cells were used to evaluate the apparent $k_{off}$ for (ICAM-1)$_{TC}$–(sFn)–(ICAM-1)$_{EC}$ bonds, (αvβ3)$_{TC}$–(sFn)–(ICAM-1)$_{EC}$ bonds, or the combination of these two bonds. When sFn (made from 0.053 or 2 U/ml thrombin) instead of sFg was introduced, the bindings became stronger as $k_{off}$ dropped from 9.8 sec$^{-1}$ to 6.3 or 3.8 sec$^{-1}$ (Fig. 6D). Thrombin alone had no effect on melanoma bond affinity, as the value of $k_{off}$ of sFg-initiated bonds was not reduced upon exposure of A375M melanoma cells to 2 U/ml thrombin ($k_{off}$ 9.8 sec$^{-1}$ for sFg vs. 10.8 sec$^{-1}$ for 2 U/ml thrombin+sFg). To determine whether ICAM-1 binding sites for sFg or sFn were responsible for bond strength, cells were pre-treated with
fibrinogen $\gamma$ chain 117–133 peptides prior to the tethering assay in the presence of sFn. $\gamma$ chain 117–133 pre-treatment significantly increased the $k_{off}$ of sFn-initiated bonds (3.8 vs 8.7 sec$^{-1}$) (Fig. 6D).

$k_{off}$ values of $\alpha\beta_3$-mediated bonds (by blocking ICAM-1; Fig. 6E) and of ICAM-1-mediated bonds (by blocking $\alpha\beta_3$; Fig. 6F) were augmented with an increase in shear rates. The value of $k_{off}$ for $\alpha\beta_3$ was increased by 46% at 200 sec$^{-1}$, while that for ICAM-1 was only increased by 29% (Fig. 6E-F). Also, at 62.5 sec$^{-1}$, $\alpha\beta_3$-initiated bonds had a $k_{off}$ of 8.6 sec$^{-1}$, which was much smaller than $k_{off}$ of ICAM-1, 14.2 sec$^{-1}$. Since the dissociation rate reflects the lifetime of the bonds, it is likely that fibrin-crosslinked (ICAM-1)$_{TC}$–(sFn)–(ICAM-1)$_{EC}$ bonds were more prone to dissociation than ($\alpha\beta_3$)$_{TC}$–(sFn)–(ICAM-1)$_{EC}$ bonds. High shear rates exerted a larger tensile force on the bonds, which resulted in an increased bond dissociation rate (Fig. 6E and F). These two sFn crosslinked bonds had similar $k_{off}$ values of 18.3 and 18.4 sec$^{-1}$ at the shear rate of 200 sec$^{-1}$, which might imply that these bonds contribute equally to melanoma adhesion to the EC through a sFn-crosslinking mechanism at high shear rates. The values of $k_{off}$ measured for ($\alpha\beta_3$)$_{TC}$–(sFn) or (ICAM-1)$_{TC}$–(sFn) bonds were comparable to those of previously measured dissociation rates of monocyte adhesion to the ECs (e.g., 15 sec$^{-1}$ at a shear rate of 40 sec$^{-1}$) and slightly larger than those for $\beta_2$-integrin-ICAM-1 bonds (e.g., 0.03–2.5 sec$^{-1}$) (22, 23, 67, 82). Therefore, the deviation of $k_{off}$ magnitude of sFn-mediated bonds might reflect the general behavior of divalent ligand-crosslinked bonds. The measured values most likely depend on the experimental approaches and data analyses, but these values fall in the range of P-selectin and PSGL-1 bonds (2, 63).

We also incorporated the values of $k_{off}$ from tethering experiments into a probability model (Eqns 1 and 2) to derive the apparent binding affinity $K_d$ for sFn-crosslinked bonds (Table 2). It was found that the affinity of the $\alpha\beta_3$ crosslinked bond was higher than that of ICAM-1 (2.98 vs. 2.22 sec$^{-1}$ at shear rate 62.5 sec$^{-1}$, and 3.08 vs. 1.32 sec$^{-1}$ at shear rate 200 sec$^{-1}$). Fig. 7 shows the best-fitting result for 1205Lu cells arrested on EC at shear rates of 62.5 and 200 sec$^{-1}$.

**DISCUSSION**
We have previously demonstrated that PMN-facilitated melanoma cell adhesion on EC follows a multistep scheme, where cell adhesion is initiated by LFA-1-mediated tethering onto endothelial ICAM-1 and is further stabilized by Mac-1 on activated PMNs (36, 71, 72). Elevated sFn levels are a characteristic property of the tumor microenvironment (8). Therefore, examining sFn mediated crosslinking mechanisms would generate valuable information about the complex intermolecular events in tumor cell extravasation.

In the present study, we showed that: (1) sFn serves as a divalent crosslinking ligand, tethering melanoma cells to the ECs; (2) sFn increases the force-regulated lifetime to a larger extent than sFg; (3) a high shear force has a larger impact than a low shear force on the enhancement of both long-term melanoma direct-adhesion frequency and PMN-mediated melanoma adhesion efficiency in the presence of sFn; (4) the promoting effect of sFn on melanoma cell adhesion is most apparent for initial tumor cell capture and firm adhesion under shear conditions; (5) ICAM-1 plays an important role in initial melanoma tethering, while αvβ3 mediates firm adhesion of melanoma cells to the endothelium. The effect of sFn is additive with that of PMN-mediated melanoma firm adhesion to the endothelium (Fig. 8).

Fibrin, by crosslinking receptors, potentiates PMN-mediated melanoma firm adhesion to EC under high shear. Elevated expression of TF, a membrane spanning procoagulant protein on melanoma cell membranes, leads to elevated levels of both sFn and fibrin polymers on primary tumor loci (51). Polymeric fibrin has been shown to form a protective sheath around circulating tumor cells (CTCs) and leads to the formation of clusters that prevent from NK (natural killer) cell invasions, facilitating CTC lodging to distant organ sites (53). Considering the vascular size restrictions and plasma skimming effects, it is less likely to find melanoma clusters formed by a fibrin clot in small capillaries. This is due to the fact that thrombin-mediated fibrin formation is a rapid event. Once thrombin is generated in plasma, fibrinogen is quickly cleaved and polymerized (11). It is difficult to determine whether fibrin monomers or fibrin clots would be more important to tether tumor cells. Our adhesion assays, which employs fibrin monomers generated by reacting sFg and thrombin in the presence of the anti-coagulant peptide GPRP, are a simplified
model system. Furthermore, it is difficult to characterize fibrin polymer-mediated binding inside a parallel-plate flow chamber since polymerization may alter the medium viscosity and affect the flow field. We have demonstrated that other proteins, including GPRP after fibrin clot removal, have no effects on either melanoma direct adhesion or PMN-mediated melanoma adhesion to the EC (Figs. 2A and 5A). To obtain more definitive evidence for sFn-dominated binding, the potential roles of other proteins in this process were excluded after the fibrin polymer was removed. This result demonstrated that applications of 2 U/ml thrombin could convert all fibrinogen to fibrin; otherwise residual fibrinogen would have increased 1205Lu adhesion. Residual thrombin did not seem to activate melanoma adhesion. This was examined in a tethering assay that showed pre-treating melanoma with 2 U/ml thrombin did not result in long-term bonds (Fig. 6D). However, another group showed that tumor-platelet aggregation was initiated by using 1-10 mU/ml thrombin (52). These results are plausible as melanoma adhesion to fibrin(ogen) proceeds through distinct receptors whose expression and affinity are sensitive to thrombin stimulation.

The tumor stroma has been characterized by the generation of tumor-derived plasminogen, which is a precursor of plasmin that can degrade fibrin to small fragments (68). These proteolytic fragments contain four RGD motifs, two located in the αC region and two in the coiled-coil connector. In the current study, an effort was made to verify these divalent binding mechanisms. When sFn was replaced with fibrinogen fragment D or plasmin-treated sFn at the same molecular level, the enhancement of melanoma adhesion was eliminated. This result emphasized the requirement of double chains for receptor-mediated adhesion. It is important to note that fragment D contains two α,β3 binding sites, one at decapeptide and the other at Aα
572–575, which might have the potential for receptor-receptor crosslinking. However, in the current study, we found no effect of fragment D on melanoma cell adhesion, particularly for long-term arrest. This may be caused by spatial hindrance, where the binding to one site masks binding to others. It is conceivable that the adhesive behavior between intact fibrin(ogen), fragment D and fragment γ chain 117-133 is different due to the ability of plasmin to cleave sFn (20).
sFn has been shown to enhance melanoma firm adhesion by 7.4-fold at 62.5 sec\(^{-1}\) and by 4-fold at 200 sec\(^{-1}\) (Fig. 1A and C) compared to sFg. However, sFn only significantly increased PMN-mediated melanoma firm adhesion at 200 sec\(^{-1}\), not at 62.5 sec\(^{-1}\) (Fig. 4A and C). This is consistent with earlier observations showing that, in contrast to selectins, fibrinogen does not have a role in the initial seeding of tumor cells within the pulmonary vasculature. Rather, fibrinogen may regulate metastasis by mediating the sustained adhesion and survival of tumor cells under high shear (54). Varying durations of tumor cell adhesion at different shear rates suggest that fibrin-initiated bonds may have substantial tensile strengths that could lead to bonds with increased lifetimes at high shear rates. Additionally, the small compliance distance may be an indication of large bond stiffness for long-term binding, which could resist large rupture forces.

It has been reported that fibrinogen binding to the lipid bilayer immobilized α\(_{\text{IIb}}\)β\(_{3}\), results in a time-dependent two-step process, where an initial reversible bond is stable and dissociated only in the presence of high levels of RGDV (39, 40, 46). It has also been reported that loading rates and contact time have a large impact on α\(_{\text{IIb}}\)β\(_{3}\) – fibrinogen rupture force (39). In the current study, we have shown that α\(_{\text{v}}\)β\(_{3}\) fibrin binding is shear rate-dependent. α\(_{\text{v}}\)β\(_{3}\) is structurally similar to α\(_{\text{IIb}}\)β\(_{3}\), whose affinity or activation state can be regulated by Mn\(^{2+}\) and activating mAbs. Therefore, the apparent force-regulated binding behavior to fibrin(ogen) might be regulated by α\(_{\text{v}}\)β\(_{3}\) activating states.

**Roles of receptors in sequential binding of melanoma to PMN and endothelial cells.** When β\(_{2}\) integrins are activated, they become high affinity receptors for sFg (20). The high affinity regions for ICAM-1 and α\(_{\text{v}}\)β\(_{3}\) on sFn are located on their γ chains. ICAM-1 expression is upregulated on the EC in response to an inflammatory microenvironment, thus becoming accessible to sFn and melanoma cells (73). Results from Fig. 1A-C suggest that both sFg and sFn increase melanoma-EC interactions under flow conditions, although sFn had a stronger ability to mediate long-term arrest at a higher shear rate. This may imply that the extra exposed binding sites on sFn modify the kinetics of the bonds (43). Earlier studies focusing on
melanoma cell adhesion to immobilized fibrinogen have revealed an \(\alpha_\beta_3\)-dependent mechanism (60).

Blocking ICAM-1 on melanoma cells reduced the frequency of tumor cell arrest on the ECs (Fig 1D), but blocking \(\alpha_\beta_3\) mainly reduced the intermediate and long-term interactions.

One solution to the limitations of cell-based flow assay is to biophysically dissect the role of each receptor at a single-bond level. As an alternative approach, we proposed a new experimental procedure and a theoretical model to extract kinetic rate constants (2, 6). Based on the tethering assays developed, we were able to show that ICAM-1 and \(\alpha_\beta_3\) had different dissociation rates at a shear rate of 62.5 sec\(^{-1}\).

The values of \(k_{off}\) measured for these bonds (i.e. \((ICAM-1)_{TC}-(sFn)-(ICAM-1)_{EC}\) and \((\alpha_\beta_3)_{TC}-(sFn)-(ICAM-1)_{EC}\)) ranging from 10 to 20 sec\(^{-1}\)) were higher than those measured for \(\beta_2\) integrin-(ICAM-1) bonds (< 1 sec\(^{-1}\)) (22, 23, 29, 37, 82), but were in line with those for selectin-ligand bonds and vWF-GPIb bonds (2, 14, 32, 41, 62, 63). This may reflect general biophysical properties of these types of divalent ligand-crosslinked bonds. To clarify this process, we assumed sFn-crosslinked bonds could dissociate in two ways (Fig. 6). For example, cell surface receptors \(A\) (e.g., ICAM-1 on EC) and \(B\) (e.g., \(\alpha_\beta_3\) on melanoma (TC)) can bind to each other via sFn in a way that:

\[
A+sFn \leftrightarrow B \xrightarrow{k_{off1}} A-sFn \leftrightarrow B \xrightarrow{k_{off2}} A-sFn \leftrightarrow B
\]  

(3)

where \(k_{on1}\) and \(k_{off1}\) are association rate and dissociation rate respectively for \(A-sFn\) bond, and \(k_{on2}\) and \(k_{off2}\) are association rate and dissociation rate for \(B-sFn\) bond, respectively. For the tethering experiments described above (assuming a single bond dissociation), the probability that a cell remains bound \((P)\) is

\[
dP/dt = -(k_{off1} + k_{off2})P \text{ with a solution, } \ln(P)/t = -(k_{off1} + k_{off2}).
\]

The apparent dissociation rate \((k_{off1} + k_{off2})\) can be determined by an unbinding curve, which plots the logarithm of the number of cells that remain bound after stop time length \(t\) according to Eqn 2 (59). We thereafter assumed that \(k_{off}\) for \((ICAM-1)-(sFn)\) bonds would be one half of the value of \(k_{off}\) for \((ICAM-1)_{TC}-(sFn)-(ICAM-1)_{EC}\) bonds (14.2 sec\(^{-1}\)) based on the crosslinking model. Therefore, \(k_{off}\) for \((ICAM-1)-(sFn)\) bonds is 7.1 sec\(^{-1}\). By subtracting \(k_{off}\) for \((ICAM-1)-(sFn)\) bonds (7.1 sec\(^{-1}\)) from \(k_{off}\) for \((\alpha_\beta_3)_{TC}-(sFn)-(ICAM-1)_{EC}\) bonds (8.6 sec\(^{-1}\)), we obtained \(k_{off}\) for \((\alpha_\beta_3)_{TC}-(sFn)\) bonds (5.5 sec\(^{-1}\)), although these values need to be further
verified by other techniques, such as atomic force microscopy or optical tweezers. We have found that
\( \alpha_3 \beta_3 \) has a larger compliance distance than ICAM-1, since its dissociation rate has larger changes than that
of ICAM-1. Therefore, ICAM-1–mediated bonds might be more compliant than \( \alpha_3 \beta_3 \)-mediated bonds,
which would make them more adaptable to shear. The distinct dissociation rates of \( \alpha_3 \beta_3 \) and ICAM-1
might clearly define their respective roles in sequential adhesion of melanoma. The critical role ICAM-1
played in initial melanoma tethering is evidenced by the reduction of melanoma adhesion frequencies for
all arrest durations following the blocking of ICAM-1 (Figs. 1D and 5D). This was further proven as the
ICAM-1 binding site, \( \gamma \) chain fibrinogen decapeptide LGGAKQAGDV, reduced the bond lifetime and
increased \( k_{off} \) (Fig. 6D).

Although we were unable to quantify the association rate and affinity directly from the tethering
assay, a kinetic model was further applied to obtain the apparent affinity by comparing the short,
intermediate and long-term arrest data (Fig. 7). The percentage of tumor cells remaining bound decreases
very rapidly in the first 0.1 sec, implying that the adhesion could most likely be mediated by only one
single bond. That is why only the events with lifetimes < 1 sec were counted for the tethering experiments
of a single bond dissociation rate measurement. In contrast, the adhesion events > 1 sec are more
important for the experiments of melanoma cells’ and PMNs’ arrests on the endothelium, which will in
turn support the extravasation.

**Possibility of involvement of other receptors and cell types.** In our study, we have focused on three
important sFn receptors: Mac-1, ICAM-1 and \( \alpha_3 \beta_3 \). Other groups reported that CD44 could also bind to
fibrin (4, 5). CD44 is a glycofucosylated molecule, which has been shown to be expressed on the surface
of colon carcinoma cells and acts as a PSGL-1-like receptor on tumor cells. In comparison, melanoma
cells only express the standard isoform of CD44 (CD44s) (83). Our current studies have shown that a
substantial decrease in melanoma cell adhesion occurs in the presence of sFn upon blocking ICAM-1 and
\( \alpha_3 \beta_3 \) (Fig. 1C), suggesting that CD44s might contribute less to melanoma tethering and firm adhesion with
the current experimental sFn concentrations and shear rates. The synergistic roles of different receptors
under shear conditions have also been studied for CD44, selectin and fibrin bonds (64). In the previously published study (52), the CD44–(P-selectin) bond was found to have a longer unstressed lifetime, which has a lower susceptibility to bond rupture under force and a higher tensile strength than CD44-fibrin bonds capable of mediating binding under higher hydrodynamic forces.

The EC monolayer used in the current studies, expressing only E-selectin and ICAM-1, is a rather simplified endothelial system for a cell adhesion study, and has limitations. For example, actual endothelial cells, like HUVECs, may express other kinds of receptors, like VCAM-1, P-selectin and αvβ3, which could bind to sFn, melanoma cells, and/or PMNs (16, 45). The local Reynolds number around the adherent cells (31) or slight cell sedimentation (79) may also affect the shear force exerted on bonds and cell adhesion frequency. Despite all these possible limitations, studying cell adhesion in a simple parallel-plate flow system still provides important insights to cell adhesive behavior under hydrodynamic shear conditions. Future studies can be carried out to elicit the contributions of other factors.

Platelets and monocytes may also play important roles in tumor cell adhesion and metastasis (10, 18, 49, 61). Other studies reported the importance of macrophages in cancer and immune recognition, focusing on the extravascular tissue space (76). However, few reports are focused on the mechanisms by which neutrophils and fibrin-mediated crosslinking potentially affect tumor cell adhesion to the endothelium within the intravascular circulation, which could significantly influence subsequent tumor cell extravasation during metastasis.

It was widely believed role of fibrin in tumor metastasis lies in its cytotoxicity protection where fibrin may polymerize and form a coat around tumors, which makes them inaccessible to immune cells. An in vitro study with plasma suggested a strong immune protective effect of sFn on tumors from natural killer cells and lymphocyte-activated killer cytotoxicity (25). In addition, sFn was shown to inhibit natural killer cell, monocyte, and lymphocyte attack, which was due to blockade of immune cell adherence to cancer cells (9, 53, 55, 56). An in vivo study suggested that fibrin is determinant component of metastatic potential (54). Fibrin could inhibit longer-term adhesion in the lung up to 1 hr without affecting short-term tumor retention. In addition, in natural killer cell-depleted mice, fibrinogen was no longer a
metastatic potential determinant (25, 55, 56). These interesting studies suggest some link between fibrin and immune killing. Although these papers disclosed the necessity of immune protective mechanism mediated by fibrinogen to facilitate tumor metastasis, they did not rule out any possible roles of fibrin-facilitated tumor cell adhesion and extravasation in metastasis. In contrast, recent studies by Konstantopoulos et al. have clearly shown an important role of fibrinogen/fibrin in mediating colon carcinoma cell adhesion, which actually supports an adhesion mechanism (4, 5, 30, 31). In addition, several in vivo studies have already reported an important role of fibrinogen and/or fibrin in platelet-mediated tumor cell adhesion in metastasis (7, 19). While the role of platelets in cancer metastasis has been widely investigated earlier, especially involving fibrinogen/fibrin, our current study has focused on how neutrophils and fibrin(ogen) modulate melanoma cell adhesion to the endothelium.

Future in vivo work needs to be conducted to fully validate the role of fibrinogen/fibrin in tumor cell adhesion and extravasation, especially for their interactions with neutrophils and the endothelium. Some recent in vivo studies have provided evidence on the role of neutrophils in mediating melanoma cell retention in the lung microvasculatures and subsequent metastasis (27, 38). In conclusion, this is the first study that characterizes soluble ligand-mediated melanoma adhesion to endothelial cells under flow conditions and determines the kinetics of specific sFn-crosslinked bonds. Delineation of biophysical interactions between fibrin(ogen) and receptors at the molecular level can shed light towards the potential development of treatment for melanoma metastasis.
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ACKNOWLEDGEMENT

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FOOTNOTES

The abbreviations used are: sLe\(^\alpha\), Sialyl Lewis X; PMN, polymorphonuclear leukocyte; sFg, soluble fibrinogen; sFn, soluble fibrin; GPRP, Gly-Pro-Arg-Pro amide; TF, tissue factor; EC, model endothelial cell, human fibroblast transfected to express ICAM-1 and E-selectin.
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FIGURE LEGENDS

Figure 1. 1205Lu cells arrest on EC is regulated by sFg/sFn, ICAM-1 and αvβ3. The assessment of melanoma cell arrests were conducted in a parallel-plate flow chamber at shear rates of 62.5 (A-B, D) and 200 (C, E) sec⁻¹, respectively. Each arrest duration was counted with time overlay on video. (A) sFn enhanced short, intermediate and long-term melanoma adhesion to EC at 62.5 sec⁻¹. (B) Blocking ICAM-1 on EC abrogated melanoma adhesion to EC via sFn. (C) sFn enhanced short, intermediate and long-term melanoma adhesion to EC at 200 sec⁻¹. *P < 0.05 compared with the “1205Lu alone” case in the same arrest duration. # P < 0.05 compared with the case “1205Lu with sFg” in the same arrest duration. Values are mean ± SE for N ≥ 3. (D-E) Relative roles of melanoma ICAM-1 and αvβ3 in sFn-mediated arrests of 1205Lu on EC as determined by functional blocking antibodies. *P < 0.05 compared with the case “1205Lu with sFg” in the same arrest duration. # P < 0.05 compared with the case “anti-ICAM-1 without sFn” in the same arrest duration. Values are mean ± SE for N ≥ 3.

Figure 2. sFn mediated melanoma adhesion to EC is dependent on a divalent binding mechanism. (A) Fibrinogen fragment D, which contains a half of α, β and γ chains, plasmin-digested sFn and residual proteins after fibrin polymer removal, failed to mediate 1205Lu intermediate and long-term adhesion to EC, although still supported short-term adhesion. (B) Thrombin and fibrinogen concentrations affect melanoma adhesion. “sFn-high”, “sFn-med”, and “sFn-low” were made respectively from 1.5mg/ml sFg + 2 U/ml thrombin; 1.5mg/ml sFg + 0.053 U/ml thrombin; and 0.015mg/ml sFg + 2 U/ml thrombin. “sFg” was 1.5mg/ml sFg without adding thrombin. *P < 0.05 compared with the case “sFn” (A) or “sFn-high” (B) respectively in the same arrest duration. Values are mean ± SE for N ≥ 3.

Figure 3. sFg or sFn affects PMN arrest on EC at different shear rates. *P < 0.05 are compared with the case “PMN” and “PMN+sFg” in the same time interval. Values are mean ± SE for N ≥ 3.
**Figure 4.** PMN-facilitated 1205Lu adhesion is affected by fibrinogen. sFn enhanced short, intermediate and long-term PMN-mediated melanoma adhesion at 62.5 sec\(^{-1}\)(A) and 200 sec\(^{-1}\) (B). (C) Blocking ICAM-1 on EC abrogated PMN-mediated 1205Lu adhesion in the presence of sFn. *P < 0.05 compared with the “1205Lu alone” case in the same arrest duration. # P < 0.05 compared with the case “1205Lu with sFg” in the same arrest duration. Values are mean ± SE for \(N \geq 3\). (D-E) Relative roles of ICAM-1 and \(\alpha_\text{v}\beta_3\) in PMN-mediated 1205Lu adhesion in the presence of sFn. *P < 0.05 compared with the “1205Lu with sFn” case in the same arrest duration. # P < 0.05 compared with the “anti-ICAM-1 without sFn” case in the same arrest duration. Values are mean ± SE for \(N \geq 3\).

**Figure 5.** PMN-mediated melanoma adhesion in the presence of sFn is dependent on a divalent binding mechanism. (A) Fibrinogen fragment D failed to mediate PMN-facilitated 1205Lu adhesion to EC. (B) Thrombin and fibrinogen concentrations affect melanoma cell adhesion. “sFn-high”, “sFn-med”, “sFn-low” were made respectively from 1.5mg/ml sFg + 2 U/ml thrombin; 1.5mg/ml sFg + 0.053 U/ml thrombin; and 0.015mg/ml sFg +2 /ml thrombin. “sFg” was 1.5mg/ml sFg without adding thrombin. *P < 0.05 compared with the case “sFn” (A) or “sFn-high” (B) respectively in the same arrest duration. Values are mean ± SE for \(N \geq 3\).

**Figure 6.** Kinetics dissociation rates (\(k_{\text{off}}\)) measured by single-bond tethering experiments. (A) Typical composite fluorescence images showing trajectories of a tethered cell during 360 msecs. Individual frames captured with Streampix(NorPix Inc.) were stacked using the ImageJ (NIH). Cell velocities became smaller when cell attached to the EC monolayer. Black arrows indicated the positions of cells in each frame. (B) The plot of cumulative distance a cell traveled tracked by Image-pro plus. The plateau reflected the duration of cell arrest. (C) Dissociation rate constant of Mac-1 binding to sFn was measured by perfusing fMLP-stimulated PMNs over sFn coated surface. (D) \(k_{\text{off}}\) values for sFn (made from 0.053 U/ml and 2 U/ml thrombin, respectively), and sFg-initiated A375M adhesive bonds. (E-F) \(k_{\text{off}}\)
values for $\alpha_v\beta_3$ (by blocking ICAM-1) and ICAM-1 (by blocking $\alpha_v\beta_3$), respectively were determined by perfusing A375M cells over EC in the presence of sFn. For first-order dissociation kinetics for transient tethers, the negative slope indicates $k_{off}$. Each data point represents the average value from three independent experiments.

**Figure 7.** Predicting the 1205Lu-endothelium binding using a kinetic model (Eqns 1 and 2). The values of dissociation rates ($k_{off}$) were adapted from tethering experiments measurement (Table 2). Percentage of 1205Lu remaining bound for $\alpha_v\beta_3$ and ICAM-1 bonds is obtained by fitting data in Fig. 1D and 1E where ICAM-1 and $\alpha_v\beta_3$ were blocked, respectively.

**Figure 8.** Schematic diagram of receptor-ligand pairings with sFn as a crosslinking molecule. When ICAM and $\alpha_v\beta_3$ bearing melanoma cells approach ICAM-1 bearing ECs and Mac-1 bearing PMNs, four types of bonds $(ICAM-1)_{TC}-(sFn)-(ICAM-1)_{EC}$, $(\alpha_v\beta_3)_{TC}-(sFn)-(ICAM-1)_{EC}$, $(ICAM-1)_{TC}-(sFn)-(Mac-1)_{PMN}$, $(\alpha_v\beta_3)_{TC}-(sFn)-(Mac-1)_{PMN}$ via freely flowing sFn molecules could form. ICAM-1-mediated bonds initiated short-term tethering, while $\alpha_v\beta_3$-mediated bonds were responsible for firm adhesion of melanoma cells to either EC or PMN.
Table 1. The expression levels of ICAM and $\alpha_{V}\beta_3$ in the cell lines used in the study

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<th>Metastatic Potential*</th>
<th>Geometric Mean Fluorescence</th>
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<tr>
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<td>ICAM-1</td>
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<td>Control IgG</td>
<td>3.7 ± 0.03</td>
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<td>1205Lu</td>
<td>++++</td>
</tr>
<tr>
<td>A375M</td>
<td>+++</td>
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*Metastatic potential was qualitatively determined from the cell line origin and adhesion potential was quantified by comparing relative mean fluorescence levels of ICAM-1 and $\alpha_{V}\beta_3$ expression by flow cytometry. Values are mean ± SE.
Table 2. Summary of apparent dissociation rate ($k_{off}$) and Affinity ($K_a$) values calculated for individual receptors-sFn bonds at shear rates of 62.5 sec$^{-1}$ and 200 sec$^{-1}$.

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<th>$k_{off}$ (sec$^{-1}$)</th>
<th>$K_a$</th>
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<tr>
<td>Mac-1</td>
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<td>2.8</td>
</tr>
</tbody>
</table>
Figure 1

A

1205Lu alone

1205Lu w/ sFg

1205Lu w/ sFn

Number of 1205Lu arrest/mm²/min

0 50 100 150 200 250

Short
Intermediate
Long

62.5 sec⁻¹

B

No blocking

EC ICAM-1 blocking

Number of 1205Lu arrest/mm²/min

0 50 100 150 200 250

Short
Intermediate
Long

EC ICAM-1 blocking

62.5 sec⁻¹

C

1205Lu alone

1205Lu w/ sFg

1205Lu w/ sFn

Number of 1205Lu arrest/mm²/min

0 50 100 150 200 250

Short
Intermediate
Long

200 sec⁻¹
D

1205Lu w/o sFn
anti-ICAM-1 w/o sFn
no blocking w/ sFn
anti-ICAM-1 w/ sFn
anti-αβ₃ w/ sFn

E

No blocking
anti-ICAM-1
anti-αβ₃

Number of 1205Lu arrest/mm²/min

62.5 sec⁻¹

200 sec⁻¹
Figure 2

A

![Graph A showing number of 1205Lu arrest/mm²/min for different treatments and time periods. The graph compares sFn, Fragment D, Plasmin-treated sFn, and Fibrin clot removal across short, intermediate, and long time periods.](image)

B

![Graph B showing number of 1205Lu arrest/mm²/min for different treatments and time periods. The graph compares sFn-high, sFg, sFn-med, and sFn-low across short, intermediate, and long time periods.](image)
Figure 3

![Graph showing the number of PMNs/mm²/min for different conditions.](image-url)
Figure 4

A

1205Lu w/ sFn

1205Lu w/ sFg

1205Lu alone

62.5 sec\(^{-1}\)

B

1205Lu w/ sFn

1205Lu w/ sFg

1205Lu alone

200 sec\(^{-1}\)

C

No blocking

EC ICAM-1 blocking

62.5 sec\(^{-1}\)
Figure 5

A

sFn
Fragment D
Plasmin-treated sFn
Fibrin clot removal

B

sFn-high
sFg
sFn-med
sFn-low

1205Lu adhesion efficiency
Figure 6

A

B

C

PMN binding to Fn

<table>
<thead>
<tr>
<th>Shear Rate</th>
<th>$k_{off}$ (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>62.5 sec$^{-1}$</td>
<td>1.6</td>
</tr>
<tr>
<td>200 sec$^{-1}$</td>
<td>2.8</td>
</tr>
</tbody>
</table>
**D**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$k_{off}$ (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sFn (by 2 U/ml thr)</td>
<td>3.8</td>
</tr>
<tr>
<td>sFn (by 0.053 U/ml thr)</td>
<td>6.3</td>
</tr>
<tr>
<td>sFg</td>
<td>9.8</td>
</tr>
<tr>
<td>2 U/ml thr+sFg</td>
<td>10.8</td>
</tr>
<tr>
<td>sFg g-chain 117-133</td>
<td>8.7</td>
</tr>
</tbody>
</table>

**E**

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<tr>
<th>Shear Rate</th>
<th>$k_{off}$ (sec$^{-1}$)</th>
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</thead>
<tbody>
<tr>
<td>62.5 sec$^{-1}$</td>
<td>14.2</td>
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<tr>
<td>200 sec$^{-1}$</td>
<td>18.3</td>
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</table>

**F**

<table>
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<tr>
<th>Shear Rate</th>
<th>$k_{off}$ (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>62.5 sec$^{-1}$</td>
<td>8.6</td>
</tr>
<tr>
<td>200 sec$^{-1}$</td>
<td>18.4</td>
</tr>
</tbody>
</table>
Figure 7

![Graph showing the percentage of 1205Lu binding over time for different conditions. The graph plots time (in seconds) on the x-axis and the percentage of binding on the y-axis. The graph includes lines for different conditions: $\alpha_\beta_3$ at 62.5 sec$^{-1}$, $\alpha_\beta_3$ at 200 sec$^{-1}$, ICAM-1 at 62.5 sec$^{-1}$, and ICAM-1 at 200 sec$^{-1}$.]
Figure 8

Bond 1: \((\text{ICAM-1})_{\text{TC}} \cdot \text{fibrin} \cdot (\text{ICAM-1})_{\text{EC}}\)
Bond 2: \((\alpha_\text{v}\beta_3)_{\text{TC}} \cdot \text{fibrin} \cdot (\text{ICAM-1})_{\text{EC}}\)
Bond 3: \((\alpha_\text{v}\beta_3)_{\text{TC}} \cdot \text{fibrin} \cdot (\text{Mac-1})_{\text{PMN}}\)
Bond 4: \((\text{ICAM-1})_{\text{TC}} \cdot \text{fibrin} \cdot (\text{Mac-1})_{\text{PMN}}\)