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

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Ozdemir T, Zhang P, Fu C, Dong C. Fibrin serves as a divalent ligand that regulates neutrophil-mediated melanoma cells adhesion to endothelium under shear conditions. Am J Physiol Cell Physiol 302: C1189–C1201, 2012. First published January 18, 2012; doi:10.1152/ajpcell.00346.2011.—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, especially integrin (α,β3) 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 cross-linking. Finally, analysis of the respective kinetics of sFn binding to Mac-1, ICAM-1, and α,β3 by single bond cell tethering assays suggested that ICAM-1 and α,β3 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.

**MELANOMA METASTASIS CONSISTS of highly regulated molecular events, including tumor detachment from the primary lesion, translocation within the blood circulation, and 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 procoagulant potential ascribed to melanoma cells has been linked to a transmembrane protein tissue factor (TF). Metastatic melanoma cells were reported to express 1,000-fold higher levels of TF on their membranes than nonmetastatic 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 (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 TCs 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 fibrinogen-receptor integrin α,β3 (60). Expression of α,β3 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 α,β3 (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, fibrinogen 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
Materials and methods

Cell preparations and reagents. A375M and 1205Lu metastatic melanoma cells (kindly provided by Dr. Gavin Robertson, Penn State Hershey Medical Center, Hershey, PA) were grown in DMEM/F12 (DMEM Nutrient Mixture F12) supplemented with 10% FBS. Adhesion molecule expressions of selected cells were detected by flow cytometry. Values are means ± SE.

Table 1. Expression levels of ICAM-1 and αvβ3 in the cell lines used in the study

<table>
<thead>
<tr>
<th>Metastatic Potential</th>
<th>ICAM-1 Mean Fluorescence</th>
<th>αvβ3 Mean Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control IgG</td>
<td>3.7 ± 0.03</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>1205Lu</td>
<td>+++++</td>
<td>165.5 ± 12.8</td>
</tr>
<tr>
<td>A375M</td>
<td>+++</td>
<td>106.7 ± 10.2</td>
</tr>
</tbody>
</table>

*Qualitatively determined from the cell line origin; adhesion potential was quantified by comparing relative mean fluorescence levels of ICAM-1 and αvβ3 expression by flow cytometry. Values are means ± SE.

Method as described by the manufacturer. Isolated PMNs were maintained at 37°C in Dulbecco’s phosphate-buffered saline containing 0.1% human serum albumin for up to a maximum of 4 h 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, University of California Davis, Davis, CA) were used in the present 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 antihuman monoclonal antibodies (mAbs) against αvβ3 (anti-CD51/61, clone 23C6) and ICAM-1 (clone BBIG-11) 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 (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 was 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 (Essex Junction, VT).

One milliliter of fresh soluble sFn (per 1 × 10^6 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 twofold concentrated sFn stock solution was mixed with cell suspension (containing melanoma cells and/or PMNs) immediately before perfusion at a 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°C 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). Then 0.02 units of plasmin per milligram fibrinogen in the presence of 1 mM CaCl2 at 37°C for 2 h 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 × 10^6 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 s⁻¹, cells were subjected to an experimental shear rate of 62.5 and 200 s⁻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.
J) 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 cross-linking adhesion mechanisms. To characterize the dynamics of these interactions, we categorized the durations of interactions (t) into short (1 s < t < 3 s), intermediate (3 s < t < 5 s), and long (t > 5 s)-term arrests. Although the binding durations of melanoma-EC interactions could be detectable as short as several milliseconds, only the longer tethers (> 1 s), which were anchored by multibonds 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 minute per millimeter squared 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 melanoma adhesion efficiency equals the number of melanoma cells arrested on the EC as a result of collision to tethered PMNs in a given duration divided by the total number of melanoma-PMN collisions. The number of cell adhesion in each case was confirmed by a second investigator who was not informed of the objective of the case 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 experiments were performed to obtain the force-regulated bond dissociation in each case was confirmed by a second investigator who was not informed of the objective of the case to ensure unbiased measurement.

The 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 s⁻¹. Compared with 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 fibrinogen-mediated melanoma binding to the EC under flow conditions.

Increasing shear rate from 62.5 to 200 s⁻¹ altered the relative contributions of sFg and sFn to the melanoma arrests (Fig. 1C). At 200 s⁻¹, sFn resulted in a marked increase in intermediate- and long-term adhesion frequencies. For intermediate-term adhesion, sFn elevated 1205Lu adhesion by twofold, while sFg did not significantly change melanoma cell adhesion (Fig. 1C, intermediate). sFn also increased the frequency of long-term adhesion by fourfold compared with sFg.

**RESULTS**

sFn 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. 1, A–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 s⁻¹).

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**Statistical analysis.** All data were obtained from at least three independent experiments and expressed by means ± SE. Statistical significance was determined using Student’s t-test or ANOVA. Tukey’s test was used in post hoc analysis for ANOVA. A 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).
The relative roles of ICAM-1 and $\alpha_\beta_3$ on melanoma cell sFn cross-linked 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 s$^{-1}$ in the presence of sFn (Fig. 1, D 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 > 50%, while blocking $\alpha_\beta_3$ had less significant effects. Blocking ICAM-1 or $\alpha_\beta_3$ prevented intermediate and long-term melanoma adhesions, suggesting ICAM-1 and $\alpha_\beta_3$ 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 $\alpha_\beta_3$ were blocked simultaneously, there were no cell arrests at both 62.5 and 200 s$^{-1}$ (data not shown).
sFg 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 ninefold (70). Therefore, to exclude the potential activation of PMNs by thrombin, PMNs were prestimulated by fMLP before being perfused into the flow chamber. To probe possible roles of sFn cross-linking in PMN adhesion to ECs, PMN tethering to the ICAM-1 was quantified. Results from Fig. 3 show that sFn increases PMN adhesion by twofold at a shear rate of 62.5 s⁻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). sFn 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. To compare the effects 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 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).
sFg significantly increased short-term PMN-mediated 1205Lu adhesion efficiency at 62.5 s⁻¹ (0.34 ± 0.020 compared with 0.20 ± 0.015 from the control) and 200 s⁻¹ (0.25 ± 0.015 compared with 0.13 ± 0.016 from the control) (Fig. 4, A 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 twofold (Fig. 4, A 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 s⁻¹, but promoted sustained adhesion at a high shear rate of 200 s⁻¹ (Fig. 4, A 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 cross-linking 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 αβ₃, respectively, on the melanoma cells and quantified the adhesion efficiency at shear rates of 62.5 and 200 s⁻¹, respectively. Results from Fig. 4, D 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 cross-linked by
sFn, while \((\alpha_\beta_1)_{TC}(sFn)-(Mac-1)_{PMN}\) 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 cross-linking 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 \(\alpha_\beta_3\). Apparent dissociation rate \(k_{off}\) for sFn receptors ICAM-1, \(\alpha_\beta_3\), 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 s\(^{-1}\) at a shear rate of 62.5 s\(^{-1}\) and 2.83 s\(^{-1}\) at 200 s\(^{-1}\) (Fig. 6C). These values fall into a range of the force-free dissociation rate of P-selectin glycoprotein ligand-1 (PSGL-1) and selectin interactions (1–10 s\(^{-1}\)) (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 s\(^{-1}\) overlapped (Fig. 6C).

A375M melanoma cells were used to evaluate the apparent \(k_{off}\) for (ICAM-1)\(_{TC}\)-(sFn)-(ICAM-1)\(_{EC}\) bonds, \((\alpha_\beta_3)_{TC}\)-(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 bonds became stronger as \(k_{off}\) dropped from 9.8 s\(^{-1}\) to 6.3 or 3.8 s\(^{-1}\) (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 \((k_{off} = 9.8\) s\(^{-1}\) for sFg vs. 10.8 s\(^{-1}\) for 2 U/ml thrombin + sFg). To determine whether ICAM-1 binding sites for sFg or sFn were responsible for bond strength, cells were pretreated with fibrinogen \(\gamma\) chain 117–133 peptides prior to the tethering assay in the presence of sFn. \(\gamma\) chain 117–133 pretreatment significantly increased the \(k_{off}\) of sFn-initiated bonds (3.8 vs. 8.7 s\(^{-1}\)) (Fig. 6D).

The \(k_{off}\) values of \((\alpha_\beta_3)_{TC}\)-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 s\(^{-1}\), while that for ICAM-1 was only increased by 29% (Fig. 6, E–F). Also, at 62.5 s\(^{-1}\), \(\alpha_\beta_3\)-initiated bonds had a \(k_{off}\) of 8.6 s\(^{-1}\), which was much smaller than \(k_{off}\) of ICAM-1, 14.2 s\(^{-1}\). Since the dissociation rate reflects the lifetime of the bonds, it is likely that fibrin-cross-linked (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. 6, E and F). These two sFn-cross-linked bonds had similar \(k_{off}\) values of 18.3 and 18.4 s\(^{-1}\) at the shear rate of 200 s\(^{-1}\), which might imply that these bonds contribute equally to melanoma adhesion to the EC through a sFn-cross-linking 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 s\(^{-1}\) at a shear rate of 40 s\(^{-1}\)) and slightly larger than those for \(\beta_3\)-integrin-ICAM-1 bonds (e.g., 0.03–2.5 s\(^{-1}\)) (22, 23, 67, 82). Therefore, the deviation of \(k_{off}\) magnitude of sFn-mediated bonds might reflect the general behavior of divalent ligand-cross-linked 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 (Eq. 1 and 2) to derive the apparent binding affinity \(K_a\) for sFg-sFn-cross-linked bonds (Table 2). It was found that the affinity of the \(\alpha_\beta_3\)-cross-linked bond was higher.
than that of ICAM-1 (2.98 vs. 2.22 s⁻¹ at shear rate 62.5 s⁻¹, and 3.08 vs. 1.32 s⁻¹ at shear rate 200 s⁻¹). Fig. 7 shows the best-fitting result for 1205Lu cells arrested on EC at shear rates of 62.5 and 200 s⁻¹.

DISCUSSION

We have previously demonstrated that PMN-facilitated melanoma cell adhesion on EC follows a multistep scheme, where
Table 2. Summary of apparent dissociation rate ($k_{off}$) and affinity ($K_d$) values calculated for individual receptors-soluble fibrin (sFn) bonds at shear rates of 62.5 s$^{-1}$ and 200 s$^{-1}$.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>$k_{off}$ (s$^{-1}$) 62.5 s$^{-1}$</th>
<th>$k_{off}$ (s$^{-1}$) 200 s$^{-1}$</th>
<th>$K_d$ (μM) 62.5 s$^{-1}$</th>
<th>$K_d$ (μM) 200 s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM-1</td>
<td>8.6</td>
<td>16.4</td>
<td>2.22</td>
<td>1.32</td>
</tr>
<tr>
<td>αββ3</td>
<td>14.2</td>
<td>18.3</td>
<td>3.98</td>
<td>3.08</td>
</tr>
<tr>
<td>Mac-1</td>
<td>1.6</td>
<td>2.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mac-1: CD11b/CD18.

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 cross-linking mechanisms would generate valuable information about the complex intermolecular events in TC extravasation.

In the present study, we showed that: 1) sFn serves as a divalent cross-linking 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 TC capture and firm adhesion under shear conditions; and 5) ICAM-1 plays an important role in initial melanoma tethering, while αββ3 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 cross-linking 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 TCs and leads to the formation of clusters that prevent from natural killer cell invasions, facilitating circulating TCs 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 TCs. Our adhesion assays, which employ fibrin monomers generated by reacting sFg and thrombin in the presence of the anticoagulant 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 pretreating 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 mM/mL thrombin (52). These results are plausible as melanoma adhesion to fibrinogen 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 present 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 αββ3 binding sites, one at decapetide and the other at Asx 572–575, which might have the potential for receptor-receptor cross-linking. However, in the present 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 fibrinogen, 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 s$^{-1}$ and by fourfold at 200 s$^{-1}$ (Fig. 1, A and C) compared with sFg. However, sFn only significantly increased PMN-mediated melanoma firm adhesion at 200 s$^{-1}$, not at 62.5 s$^{-1}$ (Fig. 4, A 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 TCs within the pulmonary vasculature. Rather, fibrinogen may regulate metastasis by mediating the sustained adhesion and survival of TCs under high shear (54). Varying durations of TC 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 αβ3, 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 Arg-Gly-Asp-Val (RGDV) (39, 40, 46). It has also been reported that loading rates and contact time have a large impact on αβ3 fibrinogen rupture force (39). In the present study, we have shown that αβ3 fibrin binding is shear rate-dependent. αβ3 is structurally similar to αIβ1, whose affinity or activation state can be regulated by Mn2+ and activating mAbs. Therefore, the apparent force-regulated binding behavior to fibrinogen might be regulated by αβ3 activating states.

Roles of receptors in sequential binding of melanoma to PMN and endothelial cells. When β2 integrins are activated, they become high-affinity receptors for sFg (20). The high-affinity regions for ICAM-1 and αβ3 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. 1, A–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 αβ3-dependent mechanism (60). Blocking ICAM-1 on melanoma cells reduced the frequency of TC arrest on the ECs (Fig. 1D), but blocking αβ3 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 αβ3 had different dissociation rates at a shear rate of 62.5 s⁻¹. The values of koff measured for these bonds [i.e., (ICAM-1)-TC-(sFn)-(ICAM-1)EC and (αβ3)TC-(sFn)-(ICAM-1)EC, ranging from 10 to 20 s⁻¹] were higher than those measured for β3 integrin-(ICAM-1) bonds (<1 s⁻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 divergent ligand-cross-linked bonds. To clarify this process, we assumed sFn-cross-linked bonds could dissociate in two ways (Fig. 6). For example, cell surface receptors A (e.g., ICAM-1 on EC) and B [e.g., αβ3 on melanoma (TC)] can bind to each other via sFn in a way that

\[
A + sFn \rightarrow B \mathbin{\overset{k_{on1}}{\rightleftharpoons}} A - sFn \rightarrow B \mathbin{\overset{k_{on2}}{\rightleftharpoons}} A - sFn + B \quad (3)
\]

where k_{on1} and k_{on2} 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

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

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 Eq. 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 s⁻¹) based on the cross-linking model. Therefore, k_{off} for (ICAM-1)-(sFn) bonds is 7.1 s⁻¹. By subtracting k_{off} for (ICAM-1)-(sFn) bonds (7.1 s⁻¹) from k_{off} for (αβ3)TC-(sFn)-(ICAM-1)EC bonds (8.6 s⁻¹), we obtained k_{off} for (αβ3) TC-(sFn) bonds (5.5 s⁻¹), although these values need to be further verified by other
techniques, such as atomic force microscopy or optical tweezers. We have found that αvβ3 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 αvβ3-mediated bonds, which would make them more adaptable to shear. The distinct dissociation rates of αvβ3 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, γ 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 TCs remaining bound decreases very rapidly in the first 0.1 s, implying that the adhesion could most likely be mediated by only one single bond. That is why only the events with lifetimes ≤ 1 s were counted for the tethering experiments of a single bond dissociation rate measurement. In contrast, the adhesion events > 1 s 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 αvβ3. 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 TCs. In comparison, melanoma cells only express the standard isoform of CD44 (CD44s) (83). Our present studies have shown that a substantial decrease in melanoma cell adhesion occurs in the presence of sFn upon blocking ICAM-1 and αvβ3 (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 a previously published study (52), the CD44-(P-selectin) bond was found to have a longer untrapped 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 present 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 of 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 TC 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 cross-linking potentially affect TC adhesion to the endothelium within the intravascular circulation, which could significantly influence subsequent TC extravasation during metastasis.

It was widely believed that the 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 a determinant component of metastatic potential (54). Fibrin could inhibit longer-term adhesion in the lung up to 1 h 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 TC adhesion and extravasation in metastasis. In contrast, recent studies by Konstantopoulos and colleagues (4, 5, 30, 31) have clearly shown an important role of fibrinogen/fibrin in mediating colon carcinoma cell adhesion, which actually supports an adhesion mechanism. In addition, several in vivo studies have already reported an important role of fibrinogen and/or fibrin in platelet-mediated TC adhesion in metastasis (7, 19). While the role of platelets in cancer metastasis has been widely investigated earlier, especially involving fibrinogen/fibrin, our present study has focused on how neutrophils and fibrinogen modulate melanoma cell adhesion to the endothelium.

Future in vivo work needs to be conducted to fully validate the role of fibrinogen/fibrin in TC 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-cross-linked bonds. Delineation of biophysical interactions between fibrinogen and receptors at the molecular level can shed light toward the potential development of treatment for melanoma metastasis.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

T.O. and P.Z. performed experiments; T.O., P.Z., and C.F. drafted manuscript; T.O., P.Z., C.F., and C.D. edited and revised manuscript; T.O., P.Z., C.F., and C.D. approved final version of manuscript; T.O., P.Z., and C.D. conception and design of research.

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