Ligand Density Dramatically Affects Integrin αIIbβ3-Mediated Platelet Signaling and Spreading.

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Abstract

The impact of ligand density on integrin-mediated cell adhesion and outside-in signaling is not well understood. Using total internal reflection fluorescent microscopy, conformation-specific antibodies and Ca\textsuperscript{2+} flux measurements, we found that the surface density of fibrinogen affects \(\alpha\text{IIb}\beta\text{3}\)-mediated platelet signaling, adhesion, and spreading. Adhesion to fibrinogen immobilized at low-density leads to rapid increases in cytosolic Ca\textsuperscript{2+} and sequential formation of filopodia and lamellipodia. In contrast, adhesion to high-density fibrinogen results in transient or no increases in Ca\textsuperscript{2+} and simultaneous formation of filopodia and lamellipodia. \(\alpha\text{IIb}\beta\text{3}\) receptors at the basal surface of platelets engage fibrinogen in a ring-like pattern at the cell edges under both conditions. This engagement is, however, more dynamic and easily reversed on high-density fibrinogen. Src and Rac activity and actin polymerization are important for adhesion to low-density fibrinogen, whereas PKC/PI3 kinases contribute to platelet spreading on high-density fibrinogen. We conclude that two fundamentally different signaling mechanisms can be initiated by a single integrin receptor interacting with the same ligand when it is immobilized at different densities.
Introduction

Integrins mediate interaction of cells with their environment, responding to activation and inhibition signals (inside-out signaling) and transmitting information initiated by ligand-receptor interaction into the cell (outside-in signaling). These interactions are vital to gene regulation, cell migration, cell proliferation, and cellular differentiation in many processes. The most abundant platelet integrin, αIIbβ3, and its signaling have been extensively studied using both platelets and other cell model systems. Structural data on this integrin and the related integrin αVβ3 have brought new insights into its function, especially with the regard to ligand binding. αIIbβ3-mediated platelet adhesion to fibrinogen has been implicated in a number of different physiologic and pathologic processes. After vascular injury, fibrinogen binds to the damaged surface and may act as one of the proteins to which platelets adhere. This is of particular significance, since αIIbβ3 can bind to immobilized fibrinogen without prior platelet activation. As fibrinogen is also present in atherosclerotic plaque, platelet adhesion to fibrinogen may contribute to thrombus formation on ruptured or eroded plaques, or even to the atherosclerotic process itself. Fibrinogen also preferentially deposits on artificial surfaces, including those used for vascular prostheses and biomaterials. Thus platelet adhesion to fibrinogen-coated surfaces is of importance in the biocompatibility of these surfaces.

αIIbβ3 interactions with immobilized fibrinogen trigger outside-in signaling followed by filopodial extensions, development of lamellipodia, and subsequent attachment and spreading (reviewed in). Platelet spreading on fibrinogen is associated with tyrosine phosphorylation of several platelet proteins including FAK, Src, and Syk. Kinases involved in these processes have been shown to include protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), Csk, Src, and Syk kinases and other molecules.

In previous studies, αIIbβ3-mediated interactions with immobilized fibrinogen were often studied in cells not expressing αIIbβ3 endogenously and mostly at fibrinogen coating concentrations ≥ 10 µg/ml. However, we have shown that platelet spreading, activation of luminal αIIbβ3 receptors, and recruitment of additional platelets is dependent on the density of immobilized fibrinogen. The differences in platelet adhesive behavior follow a biphasic pattern, with decreased spreading and activation of luminal αIIbβ3 at fibrinogen coating concentrations > 10 µg/ml. In the present study, we analyze αIIbβ3-mediated adhesion to low-density and high-density fibrinogen and demonstrate that the density of fibrinogen affects platelet adhesion from the very beginning, resulting in differences in intracellular Ca²⁺ fluxes and the dynamics of αIIbβ3 interactions with fibrinogen. These differences are associated with differences in platelet morphology and the activation of the signaling pathways involved in platelet spreading.
Materials and Methods

Reagents

Human fibrinogen (depleted of von Willebrand factor, plasminogen, and fibronectin) was from Enzyme Research Laboratories (Inc., South Bend, IN); prostaglandin E1, bovine serum albumin (grade V), and apyrase (grade VII) from Sigma (St. Louis, MO); bisindolylmaleimide, PP2, SU6656, PP3, H-1152, Y-27632, NSC23766, and cytochalasin D from Calbiochem (La Jolla, CA); wortmannin from Biomol Research Laboratories (Plymouth Meeting, PA); Oregon Green BAPTA-1, AM, Fura Red, AM, Alexa-Fluor® 594-phalloidin, latrunculin A, Alexa-Fluor® 488, 594, and 647 from Molecular Probes (Eugene, OR). Type I collagen from the skin of lathyritic rats was prepared as previously described22.

All experiments were performed at 22°C.

Platelet preparation and adhesion assay

Gel-filtered platelets in HEPES-modified Tyrode’s buffer containing 0.35% bovine serum albumin (HBMT) were prepared from ACD anticoagulated blood as described previously23. In some experiments, platelets were incubated with vehicle (DMSO) or inhibitors of PKC (10 µM bisindolylmaleimide; 1 h incubation), PI3K (20 nM wortmannin; 10 min), Src family kinases (20 µM PP2 or SU6656; PP3 as a control; 30 min), actin polymerization (10 µM cytochalasin D or 10 µM latrunculin A; 10 min), Rho kinase (5 µM H-1152 or 10 µM Y-27632; 10 min), Rac1 (100 µM NSC23766; 10 min), or apyrase (3 U/ml; 5 min).

Fibrinogen was adsorbed at concentrations of 3 and 100 µg/ml for 1 h. In some experiments, adhesion to collagen (33 µg/ml) was studied for comparison. After adsorption, washing, and blocking with HBMT, platelets in HBMT containing 2 mM Mg²⁺ were allowed to adhere for 1 h, and further washed with HBMT containing 2 mM Mg²⁺. The number of adherent platelets was quantified by assessing the endogenous acid phosphatase activity using a pNpp buffer (0.1 M citrate, pH 5.4, 0.1% Triton X-100, 5 mM para-nitrophenylphosphate)24.

Time lapse phase-contrast video microscopy

The kinetics of platelet adhesion was observed using previously described phase-contrast time-lapse videomicroscopy23. In brief, fibrinogen was adsorbed to a polystyrene-coated microchamber and platelets were allowed to adhere for 2 h. Images were obtained concurrently from 2 channels at 2.4-min intervals using a Zeiss Axiovert microscope with Zeiss Plan-Neofluar 100x/1.3 NA oil immersion objective, and acquired with an Oma4 camera (EG&G Instruments, Princeton Applied Research, Princeton, NJ) and Image-1 software (Universal Imaging Corporation, West Chester, PA).

The surface areas covered by an adherent platelet were calculated using the Image-1 software on 2-3 random fields in 6 separate experiments (average 68 platelets on each surface/experiment).
Total Internal Reflection Fluorescence Microscopy (TIR-FM)

The settings for TIR-FM illumination through the objective (Apo 60x/1.45 NA; Olympus, Melville, NY) were essentially the same as previously described. Alexa 488-conjugated proteins were excited with the 488 nm line of an Argon laser (Melles Griot, Carlsbad, CA) reflected off a dichroic mirror (498DCLP) and collected through emission band pass filters (HQ525/50M). For simultaneous dualcolor imaging of Alexa 488/594, the emissions were collected through an emission splitter (Dual-view; Optical insights) equipped with dichroic mirrors to split the emission (550DCLP) and emission band pass filters (Alexa 488, HQ525/50M; Alexa 594, HQ580LP). All filters were obtained from Chroma Technologies Corp. (Rockingham, VT). Images were acquired with a 12-bit cooled CCD ORCA-ER camera (Hamamatsu Photonics, Hamamatsu, Japan) with a resolution of 1280 x 1024 pixels (pixel size, 6.45 µm²). For time lapse experiments, platelets were allowed to adhere in the presence of Alexa 488 or Alexa 594-conjugated 7H2 Fab or IgG (20 and 50 ng/ml, respectively) and images were acquired at 1 frame/10 s. Preliminary experiments showed no difference when either IgG or Fab fragment were used for β3 labeling. In some experiments, platelets labeled with 7H2 were allowed to adhere for 1h and then AP5-Alexa 488 was added to image ligand bound receptors. This imaging was done for 5 min only in order not to induce further spreading.

Immunofluorescence microscopy of adherent platelets

Platelets were adherent at low platelet count (30,000/µl) in order to visualize single platelet morphology. Adherent platelets were stained simultaneously with Alexa 594-7H2 (anti-β3), FITC-PAC-1 (antibody to activated αIIbβ3; BD PharMingen, San Diego, CA), and Alexa 488-AP5 (anti-β3) for 5 min and then fixed. In some experiments, platelets were fixed, permeabilized, and after washing stained for F-actin with Alexa 594-phalloidin. The specimens were imaged using either TIR-FM system as described above or a Zeiss LSM-510 confocal system with Axiovert 200 microscope (Carl Zeiss, Germany) using Plan-Apochromat 100x/1.4 NA oil DIC objective. Scanning with the 488 and 543 nm lasers was performed sequentially to avoid crosstalk between the fluorescent probes. In other experiments, platelets were incubated with mepacrine (10 µM; Sigma) for 30 min at 37°C to label dense bodies; gel-filtred to remove excess mepacrine, and then allowed to adhere to fibrinogen for 1 h. After washing, the number of dense bodies per platelet was determined and recorded as 0, 1, 2, 3, 4, or more.

Cytosolic calcium concentration in adherent platelets

Changes in cytosolic calcium concentration in platelets adhering to fibrinogen were monitored and analyzed by a dual ratiometric method using confocal microscopy (same equipment as above) as per Nesbitt et al. and Yap et al. Platelets were loaded with Oregon Green BAPTA-1, AM (1 µM) and Fura Red, AM (1.25 µM) and added to the wells. Cytosolic calcium was monitored by acquiring images simultaneously in
two fluorescence channels over a period of 140 s at 2 s intervals during the first 30 min of adhesion. Differential interference contrast (DIC) images were acquired at the same time to assure focus of imaging. Fluorescence intensities were determined using LSM-510 software (Carl Zeiss) and converted to intracellular calcium concentration $\Delta[Ca^{2+}]$ as described$^{30}$. Platelets were separated into categories based on the extent of adhesion (contact, filopodia, filopodia/lamellipodia, spread, other) to compare $Ca^{2+}$ in platelets with similar morphologies. The calcium response was defined as transient when only 1 or 2 peaks of intracellular $Ca^{2+}$ were observed during the 140 s observation period, and as sustained when 3 or more peaks were observed (Fig. 3B). Platelets from 3 independent experiments ($n \geq 25$/surface/experiment) were analyzed.

**Protein tyrosine phosphorylation in adherent platelets**

Adherent platelets were incubated for 1 min with HBMT buffer containing 1 mM Na$\text{$_3$}$VO$_4$, 10 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM PMSF, 0.1 mM leupeptin, 20 $\mu$g/ml trypsin inhibitor and 6 mM N-ethylmaleimide, followed by addition of 90 °C SDS-sample buffer. Lysates were then removed, heated to 100°C for 5 min, and subjected to SDS-PAGE and immunoblotting with a mixture of two anti-phosphotyrosine monoclonal antibodies, PY20 and PY99 (Santa Cruz Biotechnology, Santa Cruz, CA). The post-transfer gel was stained with Coomassie blue to assure equal amount of loaded protein in each lane.

For immunoprecipitations, adherent platelets were washed with HBMT buffer containing 2 mM Mg$^{2+}$ and 1 mM Na$\text{$_3$}$VO$_4$ and subsequently lysed in 1 ml of lysis buffer$^{13}$ for 30 min on ice. After centrifugation (15,000 x g, 15 min, 4°C), lysates containing equal amounts of protein were immunoprecipitated with antibody to Syk or FAK [rabbit polyclonal antibodies to Syk(C-20) and FAK(C-903), Santa Cruz Biotechnology] and Protein G-Sepharose beads. Immunoprecipitates were then analyzed by immunoblotting for phosphotyrosine content with antibodies PY20 and PY99. The membranes were then stripped and reacted with an antibody to FAK (Upstate Biotechnology, Lake Placid, NY) or Syk (N-19, Santa Cruz). Densitometry was performed to quantitatively analyze the extent of protein phosphorylation (FluorChem, AlphaInnotech; San Leandro, CA).

**ATP release from adherent platelets**

Platelets were allowed to adhere to fibrinogen-coated microtiter wells for 1 h. 50 $\mu$l of ATP substrate solution (ATPlite Luminescence Assay System, Perkin Elmer, Waltham, MA) was then added and luminescence immediately measured with Flexstation II 384 Plate Reader (Molecular Devices, Sunnyvale, CA). After this, platelet adhesion was quantified using pNpp substrate as above and measured luminescence normalized per adherent platelets in each well (Luminescence/O.D.405).

**Statistical analysis**
Data that were not normally distributed are presented as median and interquartile range (IQR); Mann-Whitney Rank Sum test was used for statistical comparison (Sigma Stat, SPSS Science, Chicago, Il). For normally distributed data, means of two sets of data were compared using Student’s t-test.
**Results**

*The density of immobilized fibrinogen affects the kinetics of platelet-ligand interactions and the morphology of adherent platelets*

A fibrinogen solution of 3 µg/ml was used to prepare surfaces with low-density fibrinogen, resulting in a surface density of ~ 80 ng/cm² or 1.8x10¹¹ molecules/cm² (Fig. S1A). High-density fibrinogen was prepared using a solution of 100 µg/ml, resulting in density of ~ 500 ng/cm² or 8.9x10¹¹ molecules/cm² (Fig. S1A). As observed by time lapse phase-contrast video microscopy, platelet adhesion and spreading on low-density fibrinogen began ~ 10 min after the addition of the platelets to the chambers and was followed by recruitment of new platelets to the adherent ones and continuous platelet spreading for up to 2 h (Fig. S1B, Video 1). In contrast, platelet adhesion to high-density fibrinogen began earlier (within 5 min of platelet addition) and there was relatively little change in spreading or morphology after 40 min (Fig. S1B, Video 1). Moreover, there was virtually no recruitment of additional layers of platelets. Also, platelets adherent to high-density fibrinogen covered significantly smaller areas than platelets adherent to low-density fibrinogen [32 ± 6 µm² vs. 50 ± 9 µm², respectively (mean ± SD); p = 0.003, n = 6]. In control experiments, platelets did not adhere to the wells treated with HBMT alone and mAb 10E531, which is specific for αIIbβ3, blocked platelet adhesion to both low- and high-density fibrinogen (not shown).

To study the effect of fibrinogen densities on the spatial and temporal distribution of integrin αIIbβ3 on the basal membrane of adhering platelets, αIIbβ3 was labeled with a β3-specific, fluorescently conjugated monoclonal antibody (mAb) 7H232 and imaged using total internal reflection fluorescence microscopy (TIR-FM). Due to high density of αIIbβ3 on the platelet surface, TIR-FM imaging of labeled αIIbβ3 allowed us to follow morphological changes of platelets in contact with the surface during the adhesion. This revealed significant differences in the kinetics of filopodia and lamellipodia formation on high- vs. low-density fibrinogen after the platelets first appeared in the evanescent field (< 200 nm from the fibrinogen coated surface) (Video 2). Filopodia started to form after ~ 40-50 s on both low- and high-density fibrinogen. However, new filopodia stopped forming earlier on low-density (median = 195 s, n = 79) than on high-density fibrinogen (305 s, n = 62; p = 0.03) (Fig. 1). Lamellipodia formation began later on platelets adhering to low- than to high-density fibrinogen (140 s vs. 110 s, p = 0.04). Once lamellipodia started to form on platelets adhering to low-density fibrinogen, formation of new filopodia ceased within 55 s. In contrast, platelets adhering to high-density fibrinogen continued to form new filopodia for 195 s after the onset of lamellipodia formation. Thus, the period during which the adhering platelets were actively extending both filopodia and lamellipodia was shorter on low-density fibrinogen (p < 0.001).

*Patterns of total and activated αIIbβ3 receptors on platelets adherent to low- and high-density fibrinogen*
Conformation specific mAbs were used to monitor the localization and activation state of αIIbβ3 60 min after the initiation of adhesion. 7H2 bound to the entire platelet surface but the staining was most intense in a thin rim on the edge of spread platelets on both low- and high-density fibrinogen (Fig. 2A). AP5, a mAb specific for a ligand-induced epitope in the PSI domain of β3, also stained spread platelets in a ring pattern, but the ring was thicker in platelets on high-density than on low-density fibrinogen. Unlike on low-density fibrinogen, not all spread platelets on high-density fibrinogen showed granulomere AP5 positive staining. Most importantly, PAC-1, a mAb that selectively recognizes activated αIIbβ3, stained spread platelets on low-density fibrinogen 4x more intensely than on high-density fibrinogen (p<0.001, n = 3; Fig. 2B). The intensity of PAC-1 staining of platelets on high-density fibrinogen could be increased by adding mAb D3, which directly activates αIIbβ3 (Fig. S2). From this we conclude that it is more likely that the αIIbβ3 receptors on platelets adherent to high-density fibrinogen are in an inactivated conformation rather than all redistributed to the basal surface and engaged with ligand.

TIR-FM imaging was employed to selectively determine the conformational state of the αIIbβ3 receptors on the basal membrane of adherent platelets. Platelets labeled with subsaturating concentrations of fluorescent 7H2-Fab were allowed to adhere for 60 min and then additionally labeled with AP5 (Fig. 2C). 7H2 labeled β3 at the basal surface of platelets in a punctate pattern (Fig. S3), suggesting that the receptors were organized into small clusters. The pattern was not affected by ligand density and did not change over time. AP5 stained nearly the entire basal surface of partially spread platelets, with accentuation at the platelet edge. On fully spread platelets, AP5 produced a pure ring-like pattern, and the ring was narrower and sharper in platelets spread on low-density fibrinogen (Fig. 2C, D). Time lapse TIR-FM imaging of platelets double labeled with 7H2 and AP5 confirmed the progression of AP5 staining from diffuse staining of the surface to more ring-like pattern as platelets underwent spreading. On fully spread platelet, the AP5 stained ring in platelets on low-density fibrinogen remained nearly unchanged over a period of 5 min (Video 3, Fig. 2E). In contrast, on high-density fibrinogen, the AP5-labeling pattern was more dynamic, with movement both within the thick, mobile ring around the edges of spread platelets, and extending radially outward beyond the initial positions (Video 3). The corresponding 7H2 staining, also dynamic, did not demonstrate radial movement (Fig. 2E).

These observations suggested that αIIbβ3 interactions with high-density fibrinogen are more dynamic than with low-density fibrinogen. To test this hypothesis, platelets adherent for 60 min were treated with αIIbβ3 antagonists (the mAb c7E3 Fab or the non-peptide RGD mimetic tirofiban) or EDTA and reversal of adhesion was measured. In fact, twice as many platelets adherent to high-density fibrinogen were detached by either the αIIbβ3 antagonists or EDTA (Table I).

To assess whether any other integrin receptors contribute to the differences in platelet adhesion, we used mAbs LM609 (anti-αVβ3), BIIG2 (anti-α5), and AIIB2 (anti-β1), which selectively block ligand
binding to their respective targets. At concentrations known to inhibit ligand binding (5 – 20 µg/ml), none of these antibodies affected either the number or spreading of adherent platelets (data not shown).

**Platelet adhesion to low-, but not to high-density fibrinogen elicits rapid and sustained intracellular calcium responses**

Intracellular calcium flux during the first 30 min of platelet adhesion was measured using a dual ratiometric method. As previously reported, adhesion to fibrinogen triggered changes in intracellular calcium concentration; however, the frequency of occurrence and character of the intracellular Ca\(^{2+}\) response varied according to the fibrinogen density (Fig. 3, Video 4). Within 30 min of the initiation of adhesion to low-density fibrinogen, 90% of adherent platelets demonstrated some increase in intracellular calcium and in 40% platelets the Ca\(^{2+}\) oscillations were sustained (Fig. 3A). In contrast, only 50% of platelets adhering to high-density fibrinogen demonstrated any increase in cytoplasmic Ca\(^{2+}\) and in the vast majority of responding platelets the increase was only transient (Fig. 3A). The amplitude of the individual Ca\(^{2+}\) signals in platelets was, however, independent of the density of fibrinogen (Fig 3B). The time to onset of sustained Ca\(^{2+}\) oscillation also differed in platelets adhering to low- vs. high-density fibrinogen. Thus, on low-density fibrinogen, 8% of adhering platelets developed sustained Ca\(^{2+}\) oscillations immediately after the initial interaction with the surface, whereas on high-density fibrinogen, sustained Ca\(^{2+}\) oscillations developed only after platelets extended both filopodia and lamellipodia. Thus, platelet interaction with low-density fibrinogen triggers intracellular Ca\(^{2+}\) response more rapidly, more often, and in a more sustained manner than platelet interaction with high-density fibrinogen.

**Platelet adhesion to low-density fibrinogen leads to more intense protein tyrosine phosphorylation**

To further understand how fibrinogen surface density affects intracellular signaling, we studied its effect on tyrosine phosphorylation. The intensity of anti-phosphotyrosine staining of proteins in adherent platelets varied with the fibrinogen density, with less intensity in platelets adherent to high-density fibrinogen (Fig. 4A). Densitometric analysis demonstrated that the intensity of staining of a tyrosine-phosphorylated protein of Mr ~ 100 kDa, which was previously identified in platelets adherent to fibrinogen, was ~50% more in platelets adherent to low- than to high-density fibrinogen (Fig. 4A). The intensity of staining of tyrosine-phosphorylated proteins in platelets adherent to low-density fibrinogen was similar to that in the platelets adherent to collagen, a substrate recognized as among the most active in initiating platelet signaling.

Because both Syk and FAK tyrosine kinases have been implicated in signaling downstream of αIIbβ3, and have been reported to be tyrosine phosphorylated after platelet adhesion to fibrinogen, lysates of platelets adherent to collagen, or either low- or high-density fibrinogen were immunoprecipitated with antibodies specific for Syk and FAK. Indeed, proteins from platelets adherent to collagen showed the most
intense anti-phosphotyrosine staining of both FAK (Fig. 4B) and Syk (not shown). Densitometric analysis showed that the intensities of anti-phosphotyrosine staining of Fak (n = 4) and Syk (n = 5) in platelets adherent to high-density fibrinogen were 30 ± 19 % and 36 ± 18 % lower, respectively, than the intensities in platelets adherent to low-density fibrinogen (p = 0.03 and 0.01, respectively). These observations suggested that there may be different signaling mechanisms after platelet adhesion to low- vs. high-density fibrinogen.

Effect of apyrase and inhibitors of signal transduction and actin polymerization on morphology of adherent platelets and FAK tyrosine phosphorylation

Since both PKC and PI3K have been implicated in signaling downstream from αIIbβ3 after interaction with fibrinogen, we studied the effect of their inhibition on adhesion to low- and high-density fibrinogen. Bisindolylmaleimide, an inhibitor of PKC, and wortmannin, an inhibitor of PI3K, both decreased adhesion to low-density fibrinogen by ~50% (Table II), but had little effect on the morphology of adherent platelets or FAK tyrosine phosphorylation (Fig. 5). In contrast, these two inhibitors only reduced adhesion to high-density fibrinogen by ~15% but they markedly decreased platelet spreading and FAK tyrosine phosphorylation (Fig. 5).

Src family kinases have been shown to play a major role in αIIbβ3-mediated outside-in signaling on high-density fibrinogen19, however, their role in αIIbβ3 outside-in signaling on low-density fibrinogen is unknown. The Src family kinase inhibitor PP2 significantly reduced platelet adhesion to low-density fibrinogen but not to high-density fibrinogen (Table II). Platelet spreading was impaired on both low- and high-density fibrinogen in the presence of PP2 (Fig 6A). Similar results were obtained with SU6656, another Src family kinase inhibitors (not shown).

The small GTPases Rac and Rho have been implicated in filopodia and lamellipodia formation in platelets39;40 and other cells41. Neither the Rac-1 inhibitor (NSC2376642) nor the Rho kinase inhibitor (H-1152) had an affect on the number of adherent platelets (Table II); however, both Rac-1 and Rho kinase inhibitors decreased platelet spreading irrespective of the fibrinogen density (Fig. 6B). Surprisingly, the presence of Rac-1 or Rho kinase inhibitors did not decrease the intensity of FAK phosphorylation in platelets adherent to low-density fibrinogen, whereas FAK phosphorylation in platelets adherent to high-density fibrinogen was almost eliminated (Fig. 6C).

Since actin polymerization participates in platelet spreading, we studied the effect of the actin polymerization inhibitor. Cytochalasin D reduced the number of platelets adherent to low-density fibrinogen by ~80%, but did not inhibit the number of platelets adherent to high-density fibrinogen (Table II). It did, however, inhibit platelet spreading on high-density fibrinogen, and this effect correlated with inhibition of actin polymerization as judged by phalloidin staining (not shown). The morphology of the few platelets on low-density fibrinogen in the presence of cytochalasin D could not be determined because the adhesion was
weak and the remaining platelets were lost during the staining procedure. Latrunculin A, another inhibitor of actin polymerization, produced similar effects (not shown).

To assess the contribution of released endogenous ADP, previously implicated in platelet adhesion to and spreading on fibrinogen\(^4\), studies were performed in the presence of apyrase. Apyrase led to an ~40% decrease in adhesion to low-density fibrinogen and only ~15% decrease in adhesion to high-density fibrinogen (Table II). Platelet spreading, however, was reduced in platelets adherent to both low- and high-density fibrinogen (Fig. 6D). The presence of apyrase did not affect tyrosine phosphorylation of FAK in platelets adherent to low-density fibrinogen, but it nearly eliminated the small amount of FAK phosphorylation in platelets adherent to high-density fibrinogen (Fig. 6E).

**Platelet adhesion to low-density fibrinogen results in more platelet degranulation than to high-density fibrinogen, but release is not extensive on either surface**

High-density fibrinogen has previously been shown to only support minimal release reaction\(^4\). To assess whether low-density fibrinogen is a more thrombogenic surface, we measured ATP concentration in the supernatant 60 min after platelet adhesion. In each of 5 separate experiments the ATP release from the platelets adherent to low-density fibrinogen exceeded the ATP release from platelets adherent to high-density fibrinogen. Although absolute values for luminescence varied considerably from day to day and donor to donor (mean ± SD for low-density fibrinogen: 3,000 ± 2,800 and for high-density fibrinogen: 940 ± 940), the ratios of luminescence values on low- vs. high-density fibrinogen for each experiment ranged only from 2 – 5 (3.2 ± 1.5, \(p = 0.008\)). To assess the release reaction in individual platelets, we studied degranulation by imaging platelets labeled with the fluorescent amine, mepacrine, which concentrates in platelet dense bodies\(^2\). Adhesion to low-density fibrinogen for 60 min resulted in 50 ± 40% (mean ± SD; \(n = 4; p = 0.04\)) more platelets containing 0 or 1 dense bodies than adhesion to high-density fibrinogen. Similarly, adhesion to high-density fibrinogen resulted in 70 ± 30% (\(p < 0.01\)) more platelets with more than 4 dense bodies per platelet than adhesion to low-density fibrinogen. These data support the results of the ATP experiments in indicating greater dense granule release on low-density fibrinogen. Even after 60 min of platelet adhesion to low-density fibrinogen, however, the majority of adherent platelets retained some dense bodies. The addition of a thrombin receptor activating peptide (TRAP) to the adherent platelets resulted in nearly immediate release of most of the dense bodies as judged by the loss of the mepacrine staining (Fig. S4). In addition, the morphology of the platelets on high-density fibrinogen dramatically changed after addition of TRAP, with many platelets immediately extending lamellipodia and spreading further. In contrast, there was little change in morphology of platelets adherent to low-density fibrinogen.
Discussion

Binding of αIIbβ3 to immobilized fibrinogen during platelet adhesion results in outside-in signaling\(^{2,12}\), initiated perhaps by ligand-induced structural rearrangements between the β3 βA (βI-like) and hybrid domains\(^{3}\). Our data indicate that the surface concentration of fibrinogen affects both platelet adhesion and αIIbβ3-initiated outside-in signaling; resulting in differences in Ca\(^{2+}\) signaling, signal transduction mechanisms, morphology, and inside-out signaling (Fig. 7). Using TIR-FM we found that interactions of αIIbβ3 with low-density fibrinogen lead to bi-phasic morphologic changes during platelet attachment characterized by the sequential development of filopodia and lamellipodia and resulting in extensive spreading. Increases in intracellular Ca\(^{2+}\) occur rapidly in some platelets, with about half of them ultimately developing sustained Ca\(^{2+}\) oscillations. Actin polymerization and Src activation are essential for platelet attachment and full spreading, whereas Rac-1, PKC, and PI3K contribute partially. Activation of luminal αIIbβ3 receptors and recruitment of additional layers of platelets are specific for platelets adhering to low-density fibrinogen. Interestingly, even though we observed a decrease in platelet spreading when either apyrase or a Rac-1 inhibitor was used, there was no effect on the degree of FAK phosphorylation, suggesting that on low-density fibrinogen FAK activation requires ADP or Rac-1 to achieve platelet spreading or that FAK activation is largely independent of the degree of platelet spreading. All of our observations on platelet adhesion to low-density fibrinogen are consistent with a model in which initial αIIbβ3 interaction with fibrinogen results in outside-in signaling followed by inside-out signaling, luminal αIIbβ3 activation, and cytoskeletal rearrangements, with only minimal granule release.

In contrast, αIIbβ3 interactions with high-density fibrinogen lead to earlier onset of lamellipodia formation and a longer period of filopodia formation, resulting in a prolonged period when both processes occur simultaneously. Despite these active processes, platelet spreading is significantly reduced. Increases in intracellular Ca\(^{2+}\) occur less frequently and Ca\(^{2+}\) oscillations are sustained in only a small percentage of platelets. None of the signal transduction inhibitors used alone inhibited platelet attachment but PKC, PI3K, Src, and to some extent Rac-1, all contributed to platelet spreading. In contrast to platelets on low-density fibrinogen, inhibitors that decrease platelet spreading also decrease FAK phosphorylation. Time lapse TIR-FM and studies with αIIbβ3 antagonists demonstrate that αIIbβ3 interactions with high-density fibrinogen are more dynamic and reversed more easily compare to low-density fibrinogen. In addition, adhesion to high-density fibrinogen is associated with even less granule release and does not lead to activation of luminal αIIbβ3 receptors.

Our studies with conformation specific mAbs demonstrate that αIIbβ3 receptors exist in different conformations on the surface of a single platelet. Time lapse TIR-FM of platelets stained with fluorescently-labeled AP5, which preferentially reacts with ligand-bound receptors, suggests that initially receptors on the entire basal surface engage ligand, but with increasing spreading only receptors at the edge of the platelets...
remain engaged. The interaction with ligand is more dynamic on high density fibrinogen, perhaps because more ligand molecules than receptors are available\textsuperscript{21}. On low-density fibrinogen biphasic morphologic changes occur during platelet attachment, where formation of filopodia is followed by formation of lamellipodia, resulting in anisotropic spreading. Similar observations have been made with endothelial cells which tended to spread anisotropicaly on low-density and isotropicaly on high-density of RGD containing gels\textsuperscript{45}.

The more intense PAC-1 staining of platelets on low-density fibrinogen indicates that these platelets have more available activated $\alpha_{II}b\beta_3$ receptors. Since platelets on low-, but not high-density fibrinogen can recruit additional layer of platelets, we conclude that at least some of these activated receptors are on the luminal surface. The much greater initiation of sustained intracellular Ca\textsuperscript{2+} fluxes in platelets on low-density fibrinogen is likely to explain the greater $\alpha_{II}b\beta_3$ activation since calcium signaling is associated with $\alpha_{II}b\beta_3$ activation, platelet aggregation, and thrombus formation and propagation\textsuperscript{46};\textsuperscript{47}.

The effect of ligand density on signaling, mobility, and motility has been studied in other cells with similar findings, namely, low ligand density results in greater mobility and high ligand density results in inhibition of signaling\textsuperscript{48};\textsuperscript{50}. Random migration of $\alpha_{II}b\beta_3$-expressing CHO cells on fibrinogen was inhibited at ligand coating concentrations above ~ 5 $\mu$g/ml, a value comparable to the division we found between low- and high-density fibrinogen\textsuperscript{50}. Cox et al.\textsuperscript{48} found similar ligand density dependence of $\alpha_5\beta_1$-mediated adhesion of CHO cells to fibronectin and $\alpha_M\beta_2$-mediated adhesion of neutrophils to fibrinogen. Moreover, they observed less spreading and polarization of CHO cells on high fibronectin concentrations and concluded that high ligand densities induces a stop signal(s) for cell migration and polarization. The interplay of Rho family GTPases has been suggested to have a role in this process as Rac-1 is inhibited at high ligand densities\textsuperscript{48}. Similarly, in our studies, the morphology of platelets adhering to low-density fibrinogen in the presence of a Rac-1 inhibitor resembled the morphology of control platelets on high-density fibrinogen, suggesting that Rac GTPase may not be fully activated during adhesion to high-density fibrinogen. Ligand density has also been shown to affect activation of FAK, however, in these studies increased fibronectin density led to increased $\alpha_5\beta_1$-mediated FAK phosphorylation\textsuperscript{51};\textsuperscript{52}.

The effects of fibrinogen density and/or surface orientation appear to be especially important in the thrombogenicity of artificial surfaces. Fibrinogen preferentially adsorbs from plasma and mediates platelet interactions with artificial surfaces\textsuperscript{11};\textsuperscript{53}. Platelet recruitment, however, does not necessarily correlate with the total amount of surface-bound fibrinogen, but rather with the amount of fibrinogen that can be recognized by specific antibodies\textsuperscript{11};\textsuperscript{54};\textsuperscript{56}. We and others previously suggested that most fibrinogen molecules are oriented vertically on the surface when immobilized at high- and horizontally at low-density\textsuperscript{57};\textsuperscript{60}. Thus, on high-density fibrinogen, the $\alpha_{II}b\beta_3$ recognition sites on the $\gamma$-chain C-termini are likely to extend out from the surface whereas on low-density fibrinogen the horizontal orientation is likely to reduce access to the $\gamma$-chain C-
termini and/or induce conformational changes by multiple contacts between fibrinogen molecules and the surface. This may expose regions that are cryptic in solution and make them available for the interaction with the integrin, maybe contributing to the unique outside-in signaling observed on low-density fibrinogen. It has been recently proposed that both the γ-chain C-terminal sequence and the RGD sequence(s) are needed for maximal spreading of αIIbβ3-expressing CHO cells and platelets on fibrinogen.

It is also possible that fibrinogen adsorption to the surface at low-density results in the arrangement of the ligands in the form of clusters rather than as evenly spaced ligands. Clustering of the receptors mediating adhesion may facilitate lateral interactions among αIIbβ3 receptors and facilitate focal adhesion formation, the initiation of signal transduction, platelet activation and spreading. However, imaging with TIR-FM suggests that there is no obvious difference in cluster size of αIIbβ3 integrins on low- vs. high-density fibrinogen. Immobilizing fibrinogen at high density is associated with a thicker ring of AP5 staining suggesting that maybe a larger number of αIIbβ3 receptors are engaged in interaction with fibrinogen and it is possible, that this initiates an inhibitory signal that counterbalances any activating signals that are generated.

In conclusion, our data demonstrate that the morphologic differences in platelet adhesion to low- and high-density fibrinogen are paralleled by differences in signal transduction, indicating that the αIIbβ3 outside-in signaling mechanisms are fundamentally different. These data need to be considered when interpreting data on platelet adhesion to fibrinogen in vitro and in vivo. Moreover, the ability to modify platelet adhesion to a synthetic surface so that the platelet does not recruit additional layers of platelets has the potential to improve the biocompatibility of materials that come in contact with blood. In vivo, depending on the nature and extent vascular injury, the density of immobilized fibrinogen may range up to the high-density values defined in our studies, suggesting that our observations may also be relevant to both hemostasis and thrombosis.
Acknowledgements

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Abbreviations
mAb – monoclonal antibody; TIR-FM – total internal reflection fluorescence microscopy; TRAP - thrombin receptor activating peptide
References


Figure legends

Figure 1:
*Filopodia and lamellipodia formation in platelets adhering to low- and high-density fibrinogen.* Time lapse TIRF imaging of platelets adhering to low- or high-density fibrinogen in the presence of Alexa 488-7H2Fab was recorded for 30 min after the addition of platelets into the fibrinogen-coated wells (Video 2). Individual platelets were analyzed for the appearance of new filopodia and onset of lamellipodia formation, counting the time they first appeared in the evanescent field (i.e. within 200 nm of the substrate) as \( t = 0 \)s. The boxes in the figure represent the median and the 25\(^{th}\) and 75\(^{th}\) percentiles of the time to the onset and end of filopodia formation and the onset of lamellipodia formation. A total of 79 platelets were analyzed on low- and 62 platelets on high-density fibrinogen from two independent experiments; \(*p = 0.03, **p = 0.04\) low- vs. high-fibrinogen.

Figure 2:
*Patterns of total and activated \(\alpha\)IIb\(\beta\)3 in platelets adherent to low- and high-density fibrinogen.* Platelets were added to wells coated with fibrinogen and incubated for 1 h. Wells were then washed and adherent platelets incubated with fluorescently-labeled antibodies as described in Methods. A. By confocal microscopy, 7H2 and AP5 staining produced similar patterns on platelets adherent to both low- and high-density fibrinogen, with AP5 staining being more pronounced in the granulomere region in platelets on low-density fibrinogen. B. PAC-1, mAb specific for activated \(\alpha\)IIb\(\beta\)3, intensely stained the surface of spread platelets on low-density fibrinogen but, under the same conditions, stained only weakly the surface of spread platelets on high-density fibrinogen. Differential interference contrast images (DIC) are shown on the right side for comparison of the platelet morphology. C. TIR-FM of platelets double stained with AP5 and 7H2 revealed that AP5 staining on the basal surface of platelets spread on low-density fibrinogen appears in a very thin rim at the edge whereas AP5 staining of spread platelets on high-density fibrinogen is much thicker and diffuse. Cells marked with arrows are magnified in D. Bars, 10 \(\mu\)m. Images shown are representative of at least 2 independent experiments. D. TIR-FM images were analyzed for the width of AP5 staining by line scan analysis. The box plot shows the median and the 25\(^{th}\) and 75\(^{th}\) percentiles for 21 and 16 platelets on low- and high-density fibrinogen, respectively, from 3 independent experiments, \(*p < 0.001\). E. 7H2 staining of receptors on a platelet spread on high-density fibrinogen is static, whereas AP5 staining shows radial movement. Alexa 546-7H2 labeled platelets were allowed to adhere for 1 h and then Alexa 488-AP5 was added and both antibodies were imaged using TIR-FM for 5 min. The image of a platelet spread on high-density fibrinogen (Video 3) was taken at the beginning of the acquisition period (red) and then 4 min later. On both low- and high-density fibrinogen, 7H2 staining did not change between the first and last frame as demonstrated by colocalization (yellow). AP5 staining of platelet on high-density fibrinogen showed radial...
extension as judged by the appearance of strong green ring outside the red/yellow staining on the overlaid image. AP5 staining on low density fibrinogen was without a change between the first and last frame (yellow overlay).

**Figure 3:**
*Platelet adhesion to low-density fibrinogen leads to more rapid and sustained calcium response.* Platelets loaded with the calcium-sensitive dyes were added to wells coated with low- or high-density fibrinogen and the cytosolic calcium fluxes during the first 30 min of the adhesion process were recorded using confocal microscopy as described in Methods (Video 3). The adherent platelets were then analyzed according their morphology and the characteristics of their calcium responses. A. Population analysis demonstrated that most platelets adherent to high-density fibrinogen did not show any elevation in intracellular Ca$^{2+}$ (*p < 0.001 high vs. low-density fibrinogen, n = 3) whereas on low-density fibrinogen, ~40% of adherent platelets showed sustained Ca$^{2+}$ oscillation (**p = 0.01). B. Selected single platelet recordings of intracellular Ca$^{2+}$ fluxes typical of non-responsive platelet on high-density fibrinogen, a transient Ca$^{2+}$ elevation in a platelet on high-density fibrinogen, and sustained oscillatory Ca$^{2+}$ response in a platelet on low-density fibrinogen.

**Figure 4:**
*Platelet adhesion to low-density fibrinogen induces greater protein tyrosine phosphorylation than adhesion to high-density fibrinogen.* Platelets were allowed to adhere to fibrinogen- (fbg) or collagen (coll) -coated wells for 1 h. After washing, adherent platelets were lysed in a buffer containing phosphatase inhibitors as described in Methods. A. Equal amounts of protein were subjected to electrophoresis and immunoblotting with mAbs specific for phosphotyrosine. Phosphotyrosine staining of proteins was less intense in platelets adherent to high-density compared with low-density fibrinogen; a protein of Mr ~ 100 kD demonstrated ~50% less intense staining in platelets adherent to high-density than to low-density fibrinogen. For comparison, phosphotyrosine staining of proteins from platelets in suspension prior to adhesion is shown. B Equal amounts of protein lysates were used to immunoprecipitate FAK. Immunoprecipitated proteins were analyzed by immunoblotting for phosphotyrosine. Thereafter, the membranes were stripped and reanalyzed with antibody to FAK, to verify that the amounts of immunoprecipitated proteins were equal in all lanes. Results shown are representative of 3 independent experiments.

**Figure 5.**
*PKC and PI3K inhibition decreases FAK tyrosine phosphorylation and platelet spreading on high-density fibrinogen.* Platelets were incubated with bisindolylmaleimide (10 µM; A and B), wortmannin (20 nM; A and C), or vehicle (control) and then allowed to adhere to wells pre-coated with fibrinogen. A. Morphology of
platelets adherent to low-density fibrinogen did not change with treatment with bisindolylmaleimide or wortmannin. Platelets adherent to high-density fibrinogen in the presence of these inhibitors showed less spreading than the control platelets. B. and C. Presence of PKC or PI3K inhibitors led to a decrease in FAK tyrosine phosphorylation in platelets adherent to high-density fibrinogen only. Non-contiguous lanes from a single blot are shown in both B and C. Results shown are representative of 3 independent experiments.

**Figure 6.**

*Apyrase and Inhibitors of Src, Rac-1, or Rho kinase decreases platelet spreading on fibrinogen.* Platelets were allowed to adhere to fibrinogen in the presence of a Src-kinase inhibitor (20 $\mu$M PP2), a Rac-1 inhibitor (100 $\mu$M NSC23766), or a Rho kinase inhibitor (5 $\mu$M H1152) for 1 h. A and B. Adherent platelets were fixed, permeabilized, and stained with fluorescently labeled phalloidin for F-actin. The Src (A) and Rac-1 (B) inhibitor and to a lesser extent the Rho kinase inhibitor (B) led to impaired spreading of platelets adhering to both low- and high-density fibrinogen. C. Analysis of FAK immunoprecipitated from platelets adherent in the presence of Rac-1 and Rho kinase inhibitors revealed decreased phosphorylation of FAK in platelets adherent to high- but not to low-density fibrinogen. D. and E. Presence of apyrase led to less extensive spreading in platelets on both high- and low-density fibrinogen, but to decrease in FAK phosphorylation in platelets on high-density fibrinogen only. Non-contiguous lanes from a single blot are shown. Results shown are representative of 3 independent experiments.

**Figure 7**

*Summary of differences in platelets signaling and spreading during $\alpha$IIb$\beta$3-mediated interaction with low- vs. high-density fibrinogen.* Attachment of platelets to low-density fibrinogen requires activity of Src, PKC, and PI3 kinases and actin polymerization. Full platelet spreading depends on Rac-1 and Rho kinase. Platelet adhesion to low-density fibrinogen further leads to inside-out signaling, resulting in $\alpha$IIb$\beta$3 activation and recruitment of additional platelets on top of the adherent platelets. In contrast, platelet attachment to high-density fibrinogen is possible even in the presence of inhibitors of Src, PKC, PI3-kinase and actin polymerization. Platelet spreading depends on Src, PI3K, PKC, Rac-1, and Rho kinase activation. Few platelets are recruited to the adherent platelets indicating inadequate inside-out signaling or inhibition of luminal $\alpha$IIb$\beta$3 activation.
Table I. Reversibility of platelet adhesion by \(\alpha IIb\beta3\) antagonists or EDTA. Platelets were allowed to adhere to fibrinogen-coated microtiter wells for 1h, unbound platelets were removed by washing and wells were further filled with buffer (Control), mAb c7E3 Fab (10 µg/ml), EDTA (10 mM), or tirofiban (100 µM) for 1h. After washing, the residual platelet adhesion was assessed by measuring endogenous acid phosphatase activity and expressed as a percentage of platelets in the control. Results are expressed as mean ± SD; * \(p \leq 0.01\) compared to low-density fibrinogen.

<table>
<thead>
<tr>
<th>INHIBITOR</th>
<th>CONCENTRATION</th>
<th>RESIDUAL PLATELET ADHESION (% OF CONTROL)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low-Density Fibrinogen</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>High-Density Fibrinogen</td>
<td></td>
</tr>
<tr>
<td>c7E3 Fab</td>
<td>10 µg/ml</td>
<td>75 ± 3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>34 ± 13*</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>10 mM</td>
<td>66 ± 14</td>
<td>7</td>
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<tr>
<td></td>
<td></td>
<td>43 ± 21*</td>
<td></td>
</tr>
<tr>
<td>Tirofiban</td>
<td>100 µM</td>
<td>69 ± 21</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37 ± 25*</td>
<td></td>
</tr>
</tbody>
</table>
Table II. The effect of inhibitors on platelet adhesion to fibrinogen. Platelets were allowed to adhere to fibrinogen coated microtiter wells for 1h. After washing, platelet adhesion was assessed by endogenous acid phosphatase activity. Adhesion is expressed as a percentage of control adhesion and results are presented as mean ± SD (n = 3). * p<0.05 compared to control.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Dose</th>
<th>Target</th>
<th>Platelet Adhesion (% of Control)</th>
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<tr>
<td></td>
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<td>Low-Density Fibrinogen</td>
</tr>
<tr>
<td>Bisindolylmaleimide</td>
<td>10 µM</td>
<td>PKC</td>
<td>49 ± 15*</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>20 nM</td>
<td>PI3K</td>
<td>51 ± 15*</td>
</tr>
<tr>
<td>PP2</td>
<td>20 µM</td>
<td>Src kinase</td>
<td>43 ± 9*</td>
</tr>
<tr>
<td>Cytochalasin D</td>
<td>10 µM</td>
<td>Actin polymerization</td>
<td>20 ± 14*</td>
</tr>
<tr>
<td>Apyrase</td>
<td>2 U/ml</td>
<td>ADP</td>
<td>57 ± 12*</td>
</tr>
<tr>
<td>H-1152</td>
<td>5 µM</td>
<td>Rho Kinase</td>
<td>103 ± 19</td>
</tr>
<tr>
<td>NSC23766</td>
<td>100 µM</td>
<td>Rac1</td>
<td>82 ± 14</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2

A

<table>
<thead>
<tr>
<th>7H2 (red)</th>
<th>AP5 (green)</th>
<th>Merged</th>
<th>DIC</th>
</tr>
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<tbody>
<tr>
<td>Low-Density Fibrinogen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-Density Fibrinogen</td>
<td></td>
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B

<table>
<thead>
<tr>
<th>PAC-1</th>
<th>DIC</th>
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<td>High-Density Fibrinogen</td>
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C

<table>
<thead>
<tr>
<th>AP5</th>
<th>7H2</th>
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<tr>
<td>Low-Density Fibrinogen</td>
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</tr>
<tr>
<td>High-Density Fibrinogen</td>
<td></td>
</tr>
</tbody>
</table>

D

![Graph showing Thickness of Fibrinogen](image)

E

<table>
<thead>
<tr>
<th>High-Density Fibrinogen</th>
<th>Low-Density Fibrinogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>7H2</td>
<td></td>
</tr>
<tr>
<td>AP5</td>
<td></td>
</tr>
</tbody>
</table>

![Images showing First Frame, Last Frame 4 min later, Overlay](image)
Figure 3

A

B

No Response

Transient

Sustained Oscillatory

Response: None Transient Sustained

% of Adherent Platelets

High-density Brinogen Low-density Brinogen

\[\Delta[	ext{Ca}^{2+}]_{\text{mM}}\]

Time (s)
Figure 5

A

Low-Density Fibrinogen

Control

Wortmannin

Bisindolylmaleimide

High-Density Fibrinogen

10 μm

B

IP: Anti FAK
Blot: Anti-P-tyrosine

Mr (×10^3)

low fbg

high fbg

Bisindolylmaleimide: - + - +

C

IP: Anti-FAK
Blot: Anti-P-tyrosine

Mr (×10^3)

low fbg

high fbg

Wortmannin: - + - +

Blot: Anti-FAK
Figure 6

A

Low-Density Fibrinogen

High-Density Fibrinogen

Control

PP2

B

Control

Rac Inhibitor

Rho Kinase Inhibitor

Low-Density Fibrinogen

High-Density Fibrinogen

C

IP: Anti-FAK
Blot: Anti-P-tyrosine

D

Control

Apyrase

Low-Density Fibrinogen

High-Density Fibrinogen

E

IP: Anti-FAK
Blot: Anti-P-tyrosine

Apyrase:

- + - +

Blot: Anti-FAK
Figure 7

<table>
<thead>
<tr>
<th>ATTACHMENT</th>
<th>SPREADING</th>
<th>PLATELET-PLATELET INTERACTION</th>
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</thead>
<tbody>
<tr>
<td><strong>Low-Density Fibrinogen</strong></td>
<td>Src PKC PI3K Actin Polymerization</td>
<td>? Granule Release Rac-1 Rho Kinase</td>
</tr>
<tr>
<td><strong>High-Density Fibrinogen</strong></td>
<td>Proceeds even in the presence of inhibitors</td>
<td>Src PI3K, PKC Rac-1, Rho Kinase</td>
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</tbody>
</table>
Ligand density dramatically affects integrin αIIbβ3-mediated platelet signaling and spreading

Marketa Jirouskova, Jyoti K. Jaiswal and Barry S. Coller