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COLLOIDAL GOLD LABELLING OF FIBRINOGEN RECEPTORS IN EPINEPHRINE-
AND ADP-ACTIVATED PLATELET SUSPENSIONS

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Abstract

It has been generally accepted for over twenty years that epinephrine stimulates platelet aggregation without inducing shape change. However, it has been recently reported that discoid platelets are not recruited into ADP- or epinephrine-stimulated aggregates. Previous work in our laboratory has suggested that platelet shape change is necessary for the binding of fibrinogen to its surface receptor, which is a prerequisite for platelet aggregation. These studies seem to indicate that epinephrine-induced platelet aggregation does involve shape change. To investigate this possibility, the extent of shape change and fibrinogen binding in suspensions of epinephrine- and ADP-activated and control platelets was assessed by scanning electron microscopy (SEM). Platelets were incubated with 20 μ M epinephrine, 20 μ M ADP, or vehicle and labelled with 18 nm gold beads conjugated to fibrinogen or to a monoclonal antibody directed against the glycoprotein IIb/IIIa complex which comprises the fibrinogen receptor. Results indicate that shape change does occur in epinephrine-activated platelets as well as ADP-activated platelets. Although GP IIb/IIIa was shown to be present on both discoid and shape-changed, pseudopodial platelets, a significant degree of fibrinogen binding did not occur earlier than the pseudopodial stage in either activated or control suspensions. Platelet aggregation studies showed that the majority of platelets involved in aggregates had changed shape in both ADP- and epinephrine-treated platelet suspensions. These studies suggest that epinephrine- and ADP-induced platelet aggregation occurs via the exposure of fibrinogen receptors on shape-changed platelets.

KEY WORDS: Platelets, Colloidal gold, Fibrinogen receptor, Epinephrine, Shape change, Pseudopodia

Introduction

Platelet membrane glycoproteins regulate the hemostatic activities of platelets, especially those of adherence and aggregation. The fibrinogen receptor is of particular interest in platelet studies because soluble fibrinogen has been shown to be essential for platelet aggregation (13,20,35,36,38,44,50,51), and platelets deficient in the glycoproteins which comprise the fibrinogen receptor do not bind fibrinogen and do not aggregate (6,11,34,45).

Following exposure to foreign surfaces or to various activating agents, such as ADP or thrombin, platelets undergo a well-characterized activation sequence involving extensive shape change, which is correlated with cytoskeletal reorganization involving the polymerization of actin into microfilaments (2,4,5,8,19,26,53,56). We have examined relative numbers and distribution of the fibrinogen receptor on individual human platelets by labelling the receptor with colloidal gold-conjugated ligand or gold-conjugated antibodies, followed by observation using scanning electron microscopy (SEM). Thus, we have been able to study, in individual platelets, how relative receptor numbers and receptor distribution relate to cell activation state. By means of correlative high voltage transmission electron microscopy (HVEM) of whole mounts, we have observed the relationship of surface receptors to the underlying cytoskeleton (4,23,24,33).

The glycoprotein IIb/IIIa complex (GP IIb/IIIa) has been shown to serve as the platelet receptor for fibrinogen, and also apparently for other plasma proteins, such as fibronectin, which contain the RGD (arg-gly-asp) amino acid sequence (48). Our previous studies of the GP IIb/IIIa fibrinogen receptor have been carried out on surface-activated platelets, and have shown that the fibrinogen-gold labels (FGN/Au) will not bind to platelets until significant shape change has occurred (4,23,24,33). This correlates well with findings of classical radiolabelled ligand binding studies, which indicate that fibrinogen does not bind to an unactivated population of platelets (6,27,28,44,47). However, the fibrinogen receptor is present on the surface of even unactivated platelets, as is demonstrated by the binding of a monoclonal antibody (3) which is

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directed against the GP IIb/IIIa complex and has been shown to inhibit fibrinogen binding (12), and also by the binding of small fragments of the gamma chain of fibrinogen (21). In addition, it has been demonstrated that the advantage ligands with small Stokes radii have in gaining access to GP IIb/IIIa over larger ligands is decreased upon activation (10).

The results of these previous studies suggest that the shape change component of platelet activation may be a prerequisite for fibrinogen binding by acting to remove steric hindrance caused by other platelet membrane glycoproteins (33). Fibrinogen binding may also be closely associated with cytoskeletal changes coincident with shape change, which may direct a reorientation of the receptor in the plane of the membrane. Since soluble fibrinogen is required for platelet aggregation, it follows that shape change may also be a prerequisite for platelet aggregation due to a fibrinogen binding requirement.

Arguments against the proposed relationship between platelet shape change, fibrinogen binding, and aggregation stem from results which suggest that although platelets stimulated by most aggregating agents, such as ADP or thrombin, change shape prior to aggregation, platelets appear to aggregate in response to epinephrine without concurrent shape change (7,29,40,42). However, Milton and Frojmovic have recently examined the shapes of platelets in ADP- and epinephrine-aggregated stirred suspensions by the use of phase contrast microscopy (30). They observed a transient increase, followed by disappearance, of free discoid platelets throughout the course of aggregation, and an accompanying increase in numbers of free pseudopodial platelets. They concluded that shape-changed platelets were preferentially recruited into early aggregates, whether the aggregation was induced by ADP or epinephrine.

We presently report the use of SEM to examine the shapes of individual platelets activated by epinephrine and ADP to address the question of epinephrine- and ADP-induced platelet shape change. In addition, we have used colloidal gold labelling to examine binding to the fibrinogen receptor in epinephrine- and ADP-activated suspensions.

Materials and Methods

Preparation of Colloidal Gold

A stock solution of colloidal gold granules having an average diameter of 18-20 nm was prepared by reducing HAuCl_4 with trisodium citrate (14-18). A 4% solution of HAuCl_4 (0.5 ml) was added to 200 ml of deionized distilled water and brought to a boil. Freshly prepared 1% trisodium citrate (5.0 ml) was added rapidly to the boiling mixture and refluxed for 30 min. A color change from dark blue to red, indicative of the formation of monodisperse colloidal particles, occurs during heating. The resulting colloidal gold solution was stored at 4°C under sterile conditions. Prior to conjugation with protein, the pH of the solution was adjusted for

optimal protein adsorption with 0.2 N K_2CO_3 , as measured by gel-filled combination electrode (No. 9115, Orion Research, Inc., Cambridge, MA). Previous studies have shown the optimal pH to be at or slightly above the pI of the protein (3,16).

Conjugation of Protein to Colloidal Gold

The minimum concentration of protein necessary to stabilize the colloidal gold was determined by adsorption isotherms (15,18). A series of protein solutions of increasing concentration were made up to 1.0 ml in deionized distilled water and added to 5.0 ml of the gold solution at optimal pH. After 1 min. at room temperature, 1.0 ml of 10% NaCl was added to the protein-gold. Inadequate stabilization of the colloid results in flocculation of the gold granules, indicated by a color change from red to blue.

Protein in 10-50% excess of the amount required to stabilize 10 ml of the gold granules was brought to 1.0 ml with sterile filtered (0.22 μm Millipore, Type GS, Millipore Corporation, Bedford, MA) deionized distilled water. Sterile filtered (0.22 μm Millipore) colloidal gold was then added to bring the volume to 10.0 ml and mixed gently. After 5 min. at room temperature, 0.5 ml of freshly prepared and prefiltered (0.45 μm Millipore, Type HA) polyethylene glycol (MW 20,000, Sigma Chemical Co., St. Louis, MO) was added to further coat and stabilize the granules. The protein-gold was concentrated and separated from excess protein by centrifugation in polycarbonate tubes at 8700 x g for 30 min. The supernatant was discarded, and the concentrated red pool resuspended to 1.0 ml in sterile filtered (0.22 μm Millipore) protein-free Tyrode's buffer, pH 7.3.

Protein Preparation

Human fibrinogen (Sigma) was dissolved in Tris-buffered saline (TBS, 0.01 M Tris, 0.15 M NaCl), pH 7.4. Contaminating fibronectin was removed by passing the sample over an affinity column of gelatin-agarose (32). Aliquots of relatively fibronectin-free ($\leq 0.5\%$ protein by weight as determined by fibronectin ELISA and SDS-PAGE) fibrinogen of ~1.0 mg/ml were frozen in dry ice-isopropanol and stored at -70°C. Gels showed that other protein contaminants (e.g., plasmin[ogen]) were not present in appreciable amounts. Prior to use, samples were dialyzed overnight against 0.005 M NaCl and centrifuged at 15,000 x g for 20 min. to remove aggregates.

The monoclonal antibody 10E5, which is directed against the human platelet glycoprotein IIb/IIIa complex (GP IIb/IIIa) (12), was a gift of Dr. Barry Collier (State University of New York at Stony Brook). Samples of ~1.0 mg/ml were stored at 4°C.

Stock solutions of normal human IgG (Hu IgG, 5 mg/ml) and bovine serum albumin (BSA, 2-5 mg/ml) were prepared in deionized distilled water and diluted to the desired concentration. Both proteins were purchased (Sigma) as lyophilized powders, and were stored at 0°C after reconstitution.

Platelet Preparation

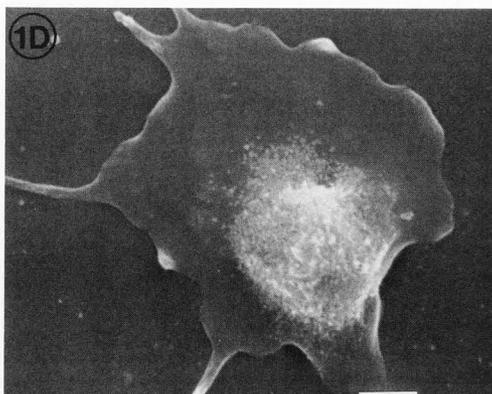
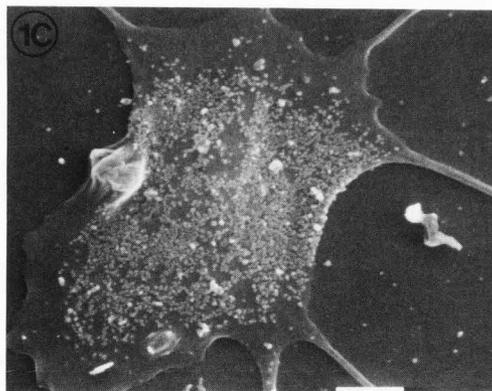
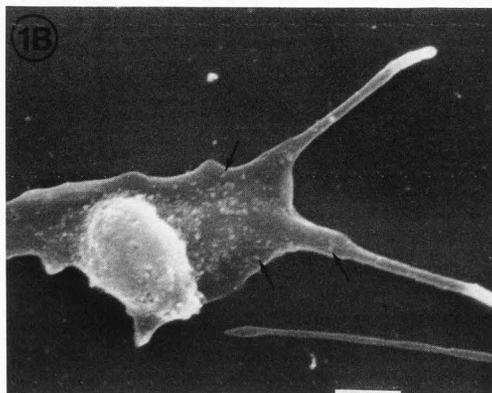
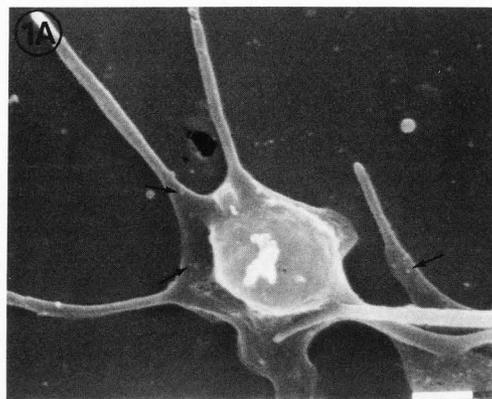
Platelets were obtained from normal healthy adult volunteers by antecubital venipuncture. Blood samples were anticoagulated with either 10 mM EGTA or 0.38% trisodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 140 x g for 15 min. at room temperature. For aggregation studies, PRP was prepared by centrifuging citrated blood at 100 x g for 10 min. in order to obtain a platelet count high enough to allow the instrument to detect platelet aggregation. Platelets were separated from plasma proteins by gel-filtration on a column of Sepharose CL-2B. The column was equilibrated with calcium-free Tyrode's buffer, pH 7.3 (136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH₂PO₄, 12 mM NaHCO₃, 2 mM MgCl₂, 5 mM dextrose, and 2 g/l BSA). Gel-filtered platelets (GFP) were collected in the void volume and were activated either in suspension or on Formvar-coated nickel Maxtaform grids (E.F. Fullman, Schenectady, NY).

Labelling the Fibrinogen Receptor

Drops of GFP were allowed to surface activate on Formvar-coated grids in a moist chamber maintained at 37°C for ~20 min. Filmed grids containing adherent platelets were rinsed in protein-free buffer after a majority of the platelets were judged to be well-spread, as observed by phase contrast microscopy. Surface-activated platelets were labelled by incubating individual grids in 20 µl drops of the appropriate gold conjugate (FGN/Au, 10E5/Au, Hu IgG/Au, or BSA/Au) for 5 min. at room temperature. The grids were then thoroughly washed in protein-free buffer and prepared for electron microscopy.

GFP were activated in suspension by incubation with 20 µM epinephrine bitartrate or 20 µM adenosine diphosphate (both from Sigma) for 5 min. at 37°C. Aliquots (100 µl) of the activated platelet suspension were then incubated with an equal volume of label, consisting of 50 µl gold-conjugated protein and 50 µl protein-free buffer supplemented with CaCl₂ to give a final Ca²⁺ concentration (in suspension of activated GFP + Ca²⁺-supplemented label) of 1 mM. Activation/labelling was stopped by addition of 10 ml of 1% glutaraldehyde in 0.1 M HEPES, pH 7.3. After a 30 min. fixation, platelets were collected by filtration on 0.4 µm pore size polycarbonate membranes (13 mm diameter Nuclepore, Nuclepore Corporation, Pleasanton, CA) and prepared for SEM.

Figure 1. Surface-activated platelets labelled with 18 nm colloidal gold conjugated to human fibrinogen (FGN/Au). Early in the spreading process (A), very few FGN/Au labels bind (arrows). As activation continues and the hyaloplasm of the platelets flattens (B), more labels bind over the entire platelet surface, including on the pseudopodia (arrows). As spreading continues, the FGN/Au labels begin to clear from the outer edges of the platelet surface (C) until they are completely centralized in the plane of the membrane, as in this nearly fully-spread form (D). Bar = 1 µm.



Aggregation Studies

Aggregation was monitored in a Payton dual channel aggregometer (Payton Scientific Inc., Buffalo, NY). GFP at $\sim 150,000/\mu\text{l}$ were supplemented with 1 mM Ca^{2+} (final concentration) and $200\ \mu\text{g/ml}$ human FGN, resulting in a final platelet count of $\sim 120,000/\mu\text{l}$. The suspensions of GFP ($500\ \mu\text{l}$) were maintained at 37°C and stirred at 1000 rpm with teflon-coated magnetic stir bars in siliconized cuvettes. Activating agents were added in small volumes (1/100 volume of GFP), and responses recorded on a dual channel Payton Aggregometer. Aggregation was stopped by the addition of 10 ml of 1% glutaraldehyde in 0.1 M HEPES, $\text{pH } 7.3$. Samples were fixed for 30 min. at room temperature, collected on filters, and prepared for SEM.

Scanning Electron Microscopy

All samples were fixed in 0.1 M HEPES-buffered 1% glutaraldehyde, $\text{pH } 7.3$, for 30 min. at room temperature, and post-fixed in 0.05% HEPES-buffered OsO_4 for 15 min. Specimens were dehydrated through a graded series of ethanol to absolute ethanol, itself dried by storage over molecular sieve. Samples were dried by the critical point procedure in an apparatus equipped with an in-line molecular sieve and a hydrophobic water-excluding filter. Samples were sputter coated with $\sim 10\text{ nm}$ gold-palladium (Auto Conductvac, SeeVac, Inc., Pittsburgh, PA) and viewed on a JEOL JSM-35C scanning electron microscope at 15 kV accelerating voltage.

Results

The binding pattern of FGN/Au to surface-activated human platelets is shown in Figures 1 A-D. Little or no labelling occurs on platelets in relatively early stages of activation. However, flattened pseudopodial platelets show extensive binding of FGN/Au over the entire surface, including out onto the pseudopodia. We have previously shown that as platelet activation and spreading continue, the FGN/Au clears from the outer edges. Finally, in fully spread platelets, FGN/Au labels are centralized in the plane of the membrane. Correlative HVEM analysis demonstrates that the individual FGN/Au labels are localized exclusively over the inner filamentous zone of the subjacent cytoskeleton (23).

In contrast to the extensive labelling observed on highly shape-changed, surface-activated platelets, platelets labelled in suspension show minimal binding of FGN/Au. Figure 2A shows the labelling of unactivated platelets incubated in suspension with FGN/Au. The majority of platelets in these samples appear to remain round or discoid, or display one short spicule. However, some platelets exhibit pseudopodia, even in the absence of a stimulating agent or surface. The FGN/Au labelling observed in unactivated suspensions appears to occur on these shape-changed, pseudopodial platelets rather than on those which are round or discoid, or which have one spicule.

A seemingly greater proportion of shape-changed platelets was observed in activated suspensions (not shown) than was observed in unactivated samples. Similarly-shaped platelets from either ADP- or epinephrine-treated samples demonstrate similar degrees of FGN/Au binding (Figures 2B and 2C). Relatively unchanged platelets do not exhibit appreciable FGN/Au labelling, as was the case with unactivated suspensions. Binding of FGN/Au occurs primarily on platelets with two or more pseudopodia, whether the activating agent is $20\ \mu\text{M}$ ADP or $20\ \mu\text{M}$ epinephrine. The FGN/Au labelling observed in all suspensions was also markedly less than that observed on the more extensively shape-changed surface-activated platelets (compare Figures 2 A-C with Figures 1C and 1D).

To test for the presence of the GP IIB/IIIa fibrinogen receptor, the binding of gold-conjugated anti-GP IIB/IIIa (10E5/Au) was examined. In unactivated platelet suspensions (Figure 3A), both round and pseudopodial platelets demonstrate considerable binding of 10E5/Au. The labelling occurs over the entire surface of the platelets, including the pseudopodia. Similarly, both round and pseudopodial platelets in suspensions activated with $20\ \mu\text{M}$ epinephrine bound 10E5/Au over the entire platelet surface (Figure 3B). No difference in 10E5/Au labelling was noted between control and epinephrine-activated suspensions. Neither surface-activated platelets nor platelet suspensions (unactivated, epinephrine-activated, or ADP-activated) incubated with Hu IgG/Au or BSA/Au demonstrated labelling.

The relationship of platelet shape change to aggregation stimulated by ADP or epinephrine was also examined. Suspensions of GFP supplemented with fibrinogen and Ca^{2+} were activated with $20\ \mu\text{M}$ ADP or $20\ \mu\text{M}$ epinephrine, and the course of aggregation was followed. HEPES-buffered glutaraldehyde was added after maximal aggregation was achieved, and the samples were processed for SEM. The resultant aggregation curves are shown in Figure 4. ADP produced more rapid and more extensive aggregation than epinephrine, as has been previously reported (39,47,50).

The shape change of both aggregated and non-aggregated platelets from these samples is shown in Figures 5 and 6. After treatment with $20\ \mu\text{M}$ ADP, all the platelets found in aggregates possess multiple pseudopodia. In addition, a large number of non-aggregated platelets are shape-changed; however, any round or discoid platelets in the sample are found among the non-aggregated population rather than involved in aggregates. Similarly, after treatment with $20\ \mu\text{M}$ epinephrine, all the aggregated platelets display multiple pseudopodia, and round or discoid platelets are found exclusively in the non-aggregated portion of the sample.

Discussion

The degree of labelling we have observed on round or discoid, and also on shape-changed, platelets with 10E5/Au indicates that the GP IIB/IIIa fibrinogen receptor is present on the

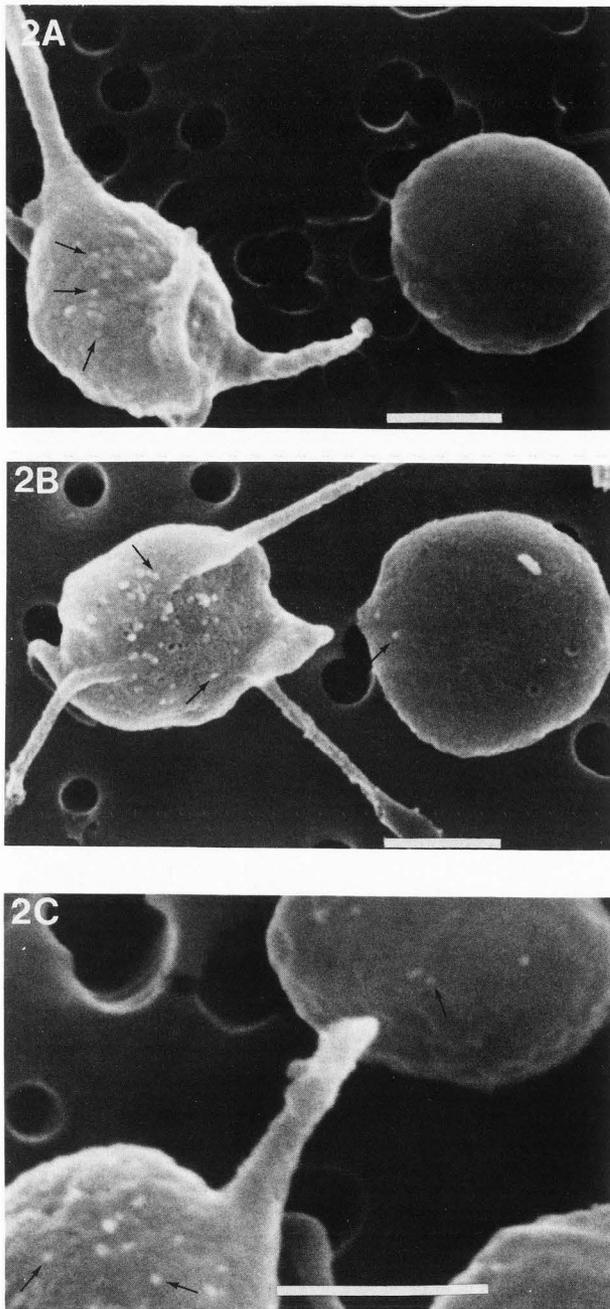


Figure 2. FGN/Au labelling of platelets activated in suspension and collected on polycarbonate filter membranes. Even in unstimulated suspensions (A), a few platelets possess pseudopodia and bind FGN/Au (arrows), whereas only an occasional label can be observed on round and discoid platelets. Following stimulation with 20 μ M ADP (B) or 20 μ M epinephrine (C), a higher proportion of platelets are shape-changed (not shown), but binding of FGN/Au (arrows) is still primarily limited to pseudopodial platelets regardless of stimulating agent. The holes in the background are pores of the filter membrane. Bar = 1 μ m.

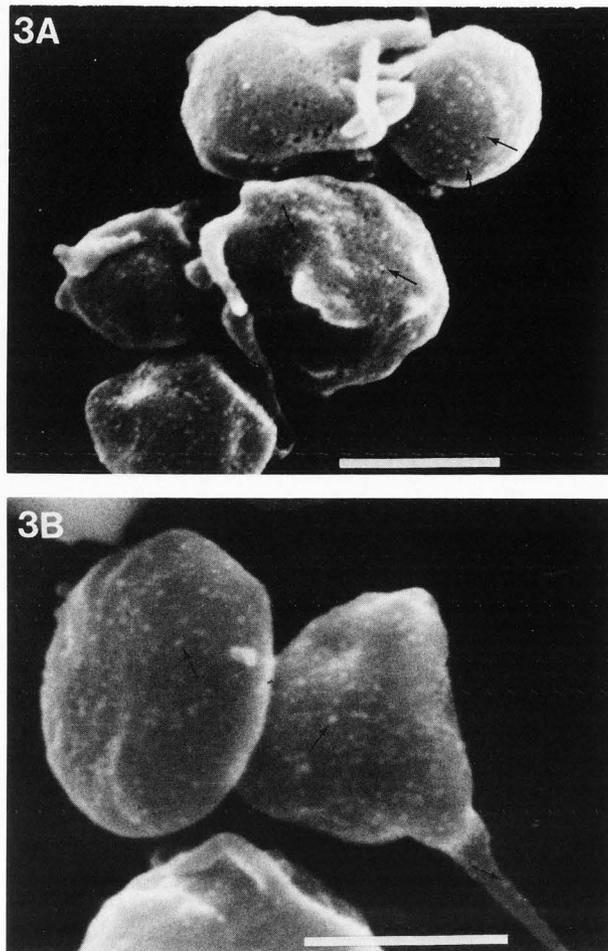


Figure 3. Platelets labelled in suspension with 18 nm colloidal gold conjugated to an antibody directed against the GP IIb/IIIa complex (10E5/Au). As also shown in Figure 2A, unactivated suspensions contain a few pseudopodial platelets (3A). However, in contrast to the labelling observed with FGN/Au, 10E5/Au (arrows) binds to both unactivated round or discoid platelets (seen at the top right of the field), and pseudopodial platelets. Following activation with 20 μ M epinephrine (3B), the labelling pattern of 10E5/Au remains the same; i.e., both unchanged and pseudopodial platelets express the GP IIb/IIIa fibrinogen receptor. Bar = 1 μ m.

surface regardless of platelet shape. Treatment of platelet suspensions with epinephrine prior to labelling does not alter the binding of 10E5/Au to individual platelets as compared to that observed in untreated samples. Thus, epinephrine activation does not cause an increase in the expression of the GP IIb/IIIa fibrinogen receptor on the platelet surface.

Binding of FGN/Au to unactivated or early activation stage platelets in suspension is sparse as compared to the FGN/Au labelling seen on highly shape-changed, late activation stage surface-activated platelets. The binding which

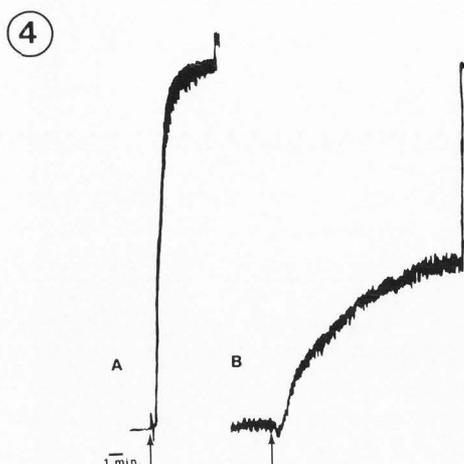


Figure 4. Aggregometer tracings of suspensions of gel-filtered platelets supplemented with 200 $\mu\text{g/ml}$ fibrinogen and 1 mM Ca^{2+} , then activated with 20 μM ADP (A) and 20 μM epinephrine (B). Time increases along the abscissa, while the ordinate scale indicates increases in light transmission. Maximum deflection was set with the Tyrode's buffer in which the platelets were suspended. Activating agents were added at the arrows. Upward deflection of the curve at the end of the plateau is due to the volume change upon the addition of HEPES-buffered glutaraldehyde to the cuvette.

does occur appears to be limited to those platelets in which the development of pseudopodia and an accompanying increase in surface area have taken place. This is the case whether the platelets are from an untreated population, or whether they are activated with ADP or epinephrine. In contrast, 10E5/Au labels unactivated and early activation stage platelets in suspension very well; the density of 10E5/Au labelling appears to be quite similar to that of FGN/Au on late and final stage surface-activated platelets. Apparently, the fibrinogen receptor is present at all stages of platelet activation, but not available to FGN/Au until a certain degree of activation has occurred.

SEM analysis of platelet suspensions fixed at the point of maximum aggregation in response to ADP (Figures 4A and 5) and epinephrine (Figures 4B and 6) clearly demonstrates that only shape-changed, pseudopodial platelets participate in aggregation. A high proportion of the non-aggregated platelets have also developed pseudopodia in both the ADP- and epinephrine-activated suspensions. However, any unchanged round or discoid platelets in the samples are found only among the non-aggregated population.

Our present study has demonstrated that the shape of platelets which are able to participate in aggregation parallels that of platelets which are able to bind FGN/Au. One can infer that the binding of fibrinogen and aggregation are related and are both preceded by shape change, regardless of stimulus. Previous results from our

laboratory have demonstrated that FGN/Au binds to spreading, dendritic-shaped surface-activated platelets, then redistributes in the plane of the membrane as activation and shape change continue to their final stages (4,23,24). The "Rainforest Hypothesis" (33) proposes that shape change must precede fibrinogen binding to platelets because the increase in membrane surface area during spreading reduces "shading" or blocking of glycoprotein receptors in the "understory" by glycoproteins in the "canopy." By increasing the distance between individual glycoprotein receptors, the "canopy" is in essence eliminated, and the GP IIb/IIIa complex which was "shaded" or blocked in unactivated platelets, becomes accessible to the large fibrinogen molecule. Molecules which are smaller and/or more flexible than fibrinogen (antibodies directed against the GP IIb/IIIa complex, fragments of the gamma chain of fibrinogen) are able to bind to unactivated platelets by going between glycoproteins of the "canopy," and therefore do not require increased surface area to gain access to receptors in the "understory." In addition to the increase in surface area during shape change, the polymerization of actin into microfilaments which follows platelet activation supports the development of pseudopodia around microfilament bundles (9,19,26,37,55,57,58). The conversion of actin from the globular to filamentous form has also been shown to result in the "tethering" of GP IIb/IIIa to the cytoskeleton (25,41,46,49), which may produce conformational changes in the receptor complex necessary for fibrinogen binding to occur.

In contrast to the current results, several previous studies have suggested that epinephrine can stimulate platelet aggregation, apparently mediated by fibrinogen binding, without producing shape change and a concomitant increase in surface area or cytoskeletal reorganization. These data have been taken as evidence against a shape change requirement for platelet aggregation or fibrinogen binding. O'Brien (40) followed platelet shape change during aggregation by assessing the amplitude of oscillations produced by a population of stirred platelets in a light path. This did not provide extensive information on the shape of individual platelets involved in aggregates. In addition, it has been shown that such turbidometric measurements are insensitive to the formation of pseudopodia (1,22,31) which are an important indicator of platelet shape change. White (54) examined individual epinephrine-aggregated platelets by transmission electron microscopy; however, the thin sections required for the work may not have reflected the overall shape of the platelets. Small pseudopodia present in sections above or below the plane of the individual sections may not have been readily detected, and the extent of pseudopod formation may have been underestimated. Therefore, although these previous studies have suggested that shape change (development of pseudopodia) is not a requirement for fibrinogen binding and platelet aggregation, the issue is not entirely resolved.

The effects of cytochalasin B (CB) on platelet shape, aggregability, and fibrinogen-binding capacity have additionally been taken as

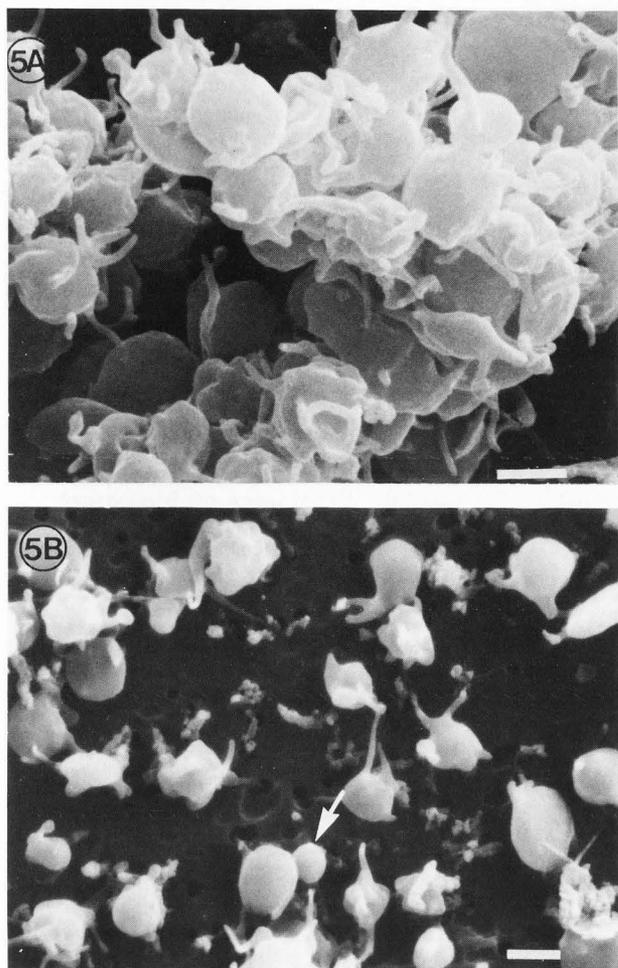


Figure 5. Platelets from the ADP-aggregated sample illustrated in Figure 4A. Platelets involved in aggregates (A) all exhibit pseudopodia. Although most of the non-aggregated platelets (B) also demonstrate shape change, any round or discoid platelets (arrow) found in the sample are found in the non-aggregated portion rather than participating in aggregates. Bar = 1 μ m.

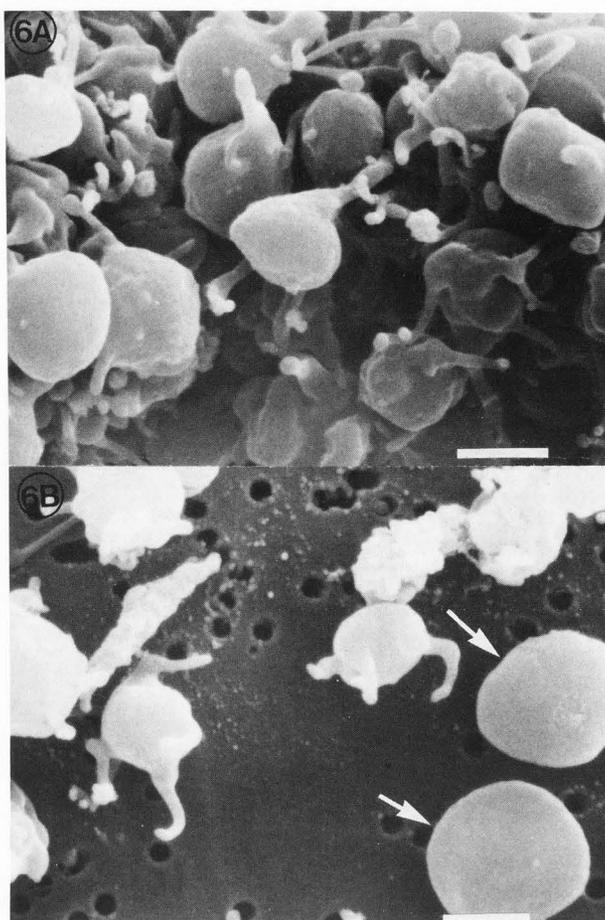


Figure 6. Platelets from the epinephrine-aggregated sample illustrated in Figure 4B. As observed in the ADP-activated samples, platelets involved in aggregates following epinephrine stimulation (A) are also exclusively those which are pseudopodial. A somewhat smaller proportion of the non-aggregated platelets in the sample demonstrate shape change. However, as with the ADP-treated sample, round and discoid platelets (arrows) are found only among the non-aggregated population rather than participating in aggregates. Bar = 1 μ m.

evidence that shape change is not required for aggregation or fibrinogen binding. It has been reported that CB-treated platelets seemingly remain discoid when aggregated by either epinephrine or ADP (54). A later study also suggested that pretreatment with CB allowed discoid platelets to aggregate in response to ADP; in addition, the amount of fibrinogen bound to CB-treated, ADP-stimulated platelets was not significantly different than the amount bound to platelets incubated with the CB vehicle (ethanol) rather than CB (43). The reasons for the increased aggregability of the non-shape-changed platelets is not clear. It is possible that CB may have directly or indirectly influenced the orientation or distribution of GP IIb/IIIa in the platelet membrane. However, it should be noted

that, as in the case of the epinephrine studies, platelet shape was evaluated by the techniques of thin sectioning and turbidometric assessment of the swirling pattern of the platelet suspension, respectively. As discussed above, these methods may not be particularly sensitive to pseudopod formation. Therefore, both the aggregation and fibrinogen binding observed in these studies could have been due to undetected shape-changed platelets. Additional work is necessary to clarify this possibility.

In support of the idea of a shape change (development of pseudopodia) requirement for platelet aggregation, Milton and Frojmovic (30) have reported that shape-changed platelets are preferentially recruited into aggregates

following stimulation with either ADP or epinephrine. Their conclusions regarding the shape of aggregated platelets were based primarily on evidence derived from assessing the shape of non-aggregated platelets at various times throughout aggregation. Their use of light microscopy (including phase contrast, darkfield, and differential-interference contrast) did not permit detailed observation of individual platelets in aggregates. Therefore, in the current study, we have employed SEM analysis of platelet shape change in response to ADP and epinephrine stimulation to provide additional evidence concerning the shape of activated and aggregated platelets, and also to explore the role of fibrinogen binding in platelet aggregation.

Although an ADP scavenger such as apyrase was not used in the epinephrine-activated samples shown here, we believe the shape change illustrated is an effect of epinephrine and is not due to ADP secreted from dense granules. In preliminary studies, we have observed that stirred suspensions which are fixed at very early time points (30 sec. or less) following exposure to epinephrine contain small (4-5 platelets) aggregates of pseudopodial platelets (not shown). The effects of ADP in epinephrine-treated suspensions are thought to be more apparent on the much larger aggregates associated with the second wave of aggregation (52). SEM analysis of epinephrine-aggregated GFP in the presence of an ADP scavenger and/or utilization of lumiaggregometry (simultaneous measurement of aggregation and secretion by means of the luciferin-luciferase system) in conjunction with SEM will be required in order to address this important issue. In addition, localization of fibrinogen on aggregated platelets, as well as investigation of the temporal relationship between fibrinogen binding and platelet aggregation, is warranted in order to more clearly elucidate the mechanism(s) underlying platelet-platelet interactions.

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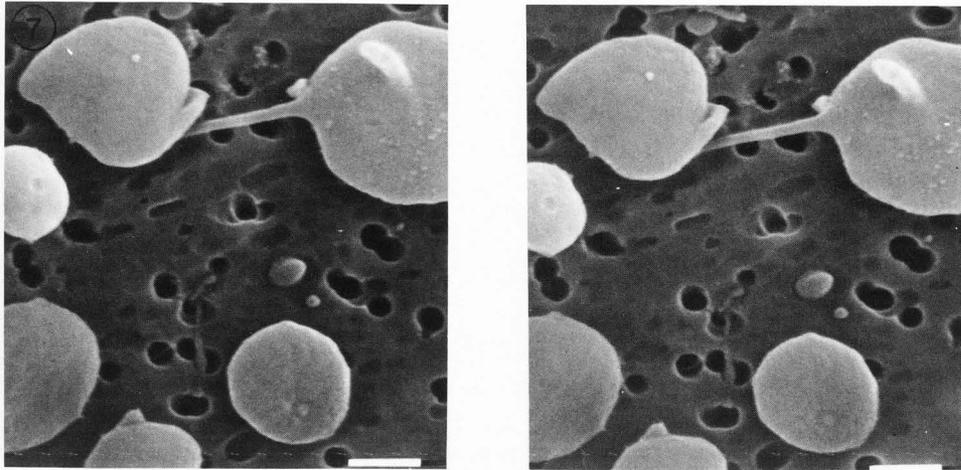
Discussion with Reviewers

M.B. Zucker: The general view is that circulating platelets are discoidal; perhaps a few have one or two pseudopods projecting from the rim of the disc. On activation, the platelets assume a more spherical configuration (though they do not have a smooth surface) and they develop more pseudopods. The platelets described here as "round" are almost certainly discs that have settled onto the surface. Do you feel that SEM is a good technique for illustrating the thickening of the discs that constitutes part of the shape change? Also, aggregates formed by epinephrine in warmed, aspirin-treated platelet-rich plasma consist largely of discs when viewed by phase contrast microscopy. As you point out in the Discussion, the platelets used in this experiment have doubtless been exposed to platelet-derived ADP. Do you feel they are suitable for studying the shape change caused by epinephrine?

Authors: We feel that our technique of studying epinephrine-induced shape change through the use of SEM is superior to the use of phase contrast microscopy. The "halo" surrounding the cell or platelet in question which occurs in phase contrast microscopy obscures the detection of small pseudopodia which may be present. This is because the modulation transfer function for phase contrast microscopy is minimal for objects in the size range of small pseudopodia. On the other hand, the modulation transfer function is nearly half maximal when these objects are viewed by differential interference contrast (DIC) microscopy. Additionally, video enhancement can be applied to boost the range of contrast obtainable when viewing objects of this size by DIC microscopy. In our own work (unpublished), we have found that warmed, aspirin-treated platelets which are activated with 20 μ M epinephrine for 5 minutes and then fixed in 1% glutaraldehyde do not appear to possess pseudopodia when viewed by phase contrast microscopy. However, platelets taken from this same sample are observed to possess multiple pseudopodia when viewed by video-enhanced DIC microscopy. Therefore, we do not feel that phase contrast microscopy is the best method for assessing the platelet shape change response. With regard to the discoid (as opposed to spherical) appearance of epinephrine-treated platelets, we believe that the important aspect of the response is not the thickening of the discs, but the development of pseudopodia. This can be determined by the use of stereo pairs of scanning electron micrographs (Figure 7) which provide a three-dimensional view of the sample. The platelets in this stereo pair are from the same sample as seen in Figure 2C. FGN/Au labels are clearly seen to select not for round over discoid platelets, but for platelets which have developed pseudopodia as opposed to those which have not.

J.F. Mustard: Epinephrine does not aggregate platelets in the presence of physiological concentrations of calcium when traces of other agonists such as ADP are

Figure 7. Stereo pair of platelets from the same sample as in Figure 2C. See text for description. Bar = 1 μ m.



unavailable. In our own work, studies that have been carried out in platelets in a low calcium medium led to an activation of the arachidonate pathway that produces aggregation which is not due to the primary stimulus, but due to the secondary effect of aggregation on the arachidonate pathway. This can be blocked by treatment of the cyclo-oxygenase with a non-steroidal anti-inflammatory drug or by making certain that the platelets are suspended in the presence of physiological concentrations of calcium and that there are no traces of other agonists such as ADP in the suspending medium. Do you feel that a likely explanation for your observed epinephrine effect is that, in a medium with low calcium, the epinephrine induced the platelets to stick transiently to each other as discs, which then led to activation of the arachidonate pathway which produced shape change in the larger aggregates (Figures 5 and 6)?

Authors: There are reports in the literature of platelets exhibiting a primary aggregation response to epinephrine after treatment with aspirin (J.S. Bennett, G. Vilaire, J.W. Burch, *J Clin Invest*, 68:981-987, 1981) and after treatment with aspirin and subsequent incubation with either apyrase or creatine phosphate/creatine phosphokinase. (E.I. Peerschke, *Blood*, 60:71-77, 1982). Although both arachidonate metabolites and secreted ADP may have played a role in the shape change observed in the large aggregates, we have noted similar shape change in the small, initial aggregates associated with primary aggregation. Since all platelet suspensions were supplemented with a physiological concentration of calcium ions, we do not feel we have stimulated the arachidonate pathway outside of the primary epinephrine stimulus. Therefore, we maintain the point that the shape of aggregated platelets (Figures 5A and 6A) parallels that of platelets which are able to bind FGN/Au.

M.M. Frojmovic: How do you know that an accompanying change in surface area has taken place with shape change? This is expected (see M.M. Frojmovic and J.G. Milton, *Physiological Reviews*, 62:185-261, 1982), especially for ADP-activated platelets, but more quantitative studies are generally required in this area.

Authors: Our claim that an increase in surface area accompanies the development of pseudopodia is based on two pieces of evidence. First, although we have not made direct morphometric measurements, the apparent size of the main body of the platelet does not decrease when pseudopodia begin to appear, as would be expected if the ratio of surface area to internal area did not increase. Second, surface area comparisons between unactivated and fully spread, surface-activated platelets have been calculated (see reference 32), and have shown that the surface area approximately doubles during the surface activation process. It is more likely that this two-fold increase occurs throughout surface activation as a continuum, much the same as the shape change process itself proceeds on a continuum, from round or discoid, to pseudopodial, spreading pseudopodial, spreading, and finally, to the fully spread shapes (see Figure 1). We agree, however, that further morphometric evaluation of this issue is required.

J.M. Gerrard: It would appear that the critical test of the Rainforest Hypothesis involves labelling and tagging other glycoproteins (e.g., glycoprotein Ib) and then looking at the distance between such glycoprotein molecules in the unactivated and activated discoid and shape-changed platelets to see if indeed there is a relative increased distance between these glycoprotein molecules in the areas where glycoprotein IIb/IIIa fibrinogen binding receptor becomes available. Have you done such studies with antibodies that would label glycoproteins which might act as a "canopy" or covering?

Authors: Actual measurement of the distance between labelled glycoprotein molecules would be difficult using the system described here because of the somewhat limited resolving power of traditional SEM. However, the recent availability of a low voltage, ultrahigh resolution (objects in the 10-20 Å range can be resolved) SEM would make such experiments feasible. It is also possible that binding of gold-conjugated native ligands will shed additional light on the proposed Rainforest Hypothesis. For instance, if glycoprotein Ib is a member of the "canopy," incubation with its native ligand, von Willebrand factor, may partially block subsequent binding of fibrinogen to GP IIb/IIIa, which we propose to be a member of the understory. On the other hand, incubation with fibrinogen should have little effect on the subsequent binding of von Willebrand factor if the model holds true.

M.B. Zucker: Your observation that much more 10E5/Au than FGN/Au binds to each activated platelet is not consistent with the results with iodine-labelled material, which indicate about the same number of binding sites on activated platelets. Do you have any explanation for this?

Authors: On fully spread, surface-activated platelets, we observe nearly equal numbers of FGN/Au and 10E5/Au labels. During the activation process, labelling with FGN/Au appears to be less dense than that seen with 10E5/Au, which labels GP IIb/IIIa very well, even on unactivated platelets. We feel that the absence of FGN/Au labelling observed on unactivated platelets, and the apparently decreased labelling of FGN/Au as compared to 10E5/Au on platelets activated in suspension, is due to steric hindrance occurring at the level of the protein molecule rather than at the level of the colloidal gold bead. However, the use of a more concentrated colloidal suspension of smaller beads for a longer period of time may more accurately depict the true situation, and permit earlier, more rapid binding of FGN/Au to receptors as they become available for fibrinogen binding.