JCI The Journal of Clinical Investigation

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J Clin Invest. 1993;92(6):2796-2806. https://doi.org/10.1172/JCI116899.

Research Article

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Studies of Activated GPIIb/IIIa Receptors on the Luminal Surface of Adherent Platelets

Paradoxical Loss of Luminal Receptors When Platelets Adhere to High Density Fibrinogen

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Abstract

The accessibility of activated GPIIb/IIIa receptors on the luminal surface of platelets adherent to damaged blood vessels or atherosclerotic plaques is likely to play a crucial role in subsequent platelet recruitment. To define better the factors involved in this process, we developed a functional assay to assess the presence of activated, luminal GPIIb/IIIa receptors, based on their ability to bind erythrocytes containing a high density of covalently coupled RGD-containing peptides (thromboerythrocytes). Platelets readily adhered to wells coated with purified type I rat skin collagen and the adherent platelets bound a dense lawn of thromboerythrocytes. With fibrinogen-coated wells, platelet adhesion increased as the fibrinogen-coating concentration increased, reaching a plateau at about 11 μg/ml. Thromboerythrocyte binding to the platelets adherent to fibrinogen showed a paradoxical response, increasing at fibrinogen coating concentrations up to ~ 4–6 μg/ml and then dramatically decreasing at higher fibrinogen-coating concentrations. Scanning electron microscopy demonstrated that the morphology of platelets adherent to collagen was similar to that of platelets adherent to low density fibrinogen, with extensive filopodia formation and ruffling. In contrast, platelets adherent to high density fibrinogen showed a bland, flattened appearance. Immunogold staining of GPIIb/IIIa receptors demonstrated concentration of the receptors on the filopodia, and depletion of receptors on the flattened portion of the platelets. Thus, there is a paradoxical loss of accessible, activated GPIIb/IIIa receptors on the luminal surface of platelets adherent to high density fibrinogen. Two factors may contribute to this result: engagement of GPIIb/IIIa receptors with fibrinogen on the abluminal surface leading to the loss of luminal receptors, and loss of luminal filopodia that interact with thromboerythrocytes. These data provide insight into the differences in thrombogenicity between surfaces, and may provide a mechanism for purposefully passivating platelet-reactive artificial surfaces. (*J. Clin. Invest.* 1993. 92:2796–2806.) Key words: platelet adhesion • glycoprotein IIb/IIIa • fibrinogen • collagen • integrin receptors

Portions of this work have appeared in abstract form (1992. *Circulation.* 86:I-683).

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Received for publication 14 May 1993 and in revised form 29 July 1993.

J. Clin. Invest.

0021-9738/93/12/2796/11 $2.00
Volume 92, December 1993, 2796–2806

Introduction

After injury to normal blood vessels or atherosclerotic lesions, platelets rapidly adhere to the damaged surface. This process is thought to be mediated by platelet receptors that have high affinity for the adhesive proteins present in normal blood vessel walls and atherosclerotic plaques (1–3). Considerable evidence supports an important role for the interactions between platelet GPIb with vWf (1–6) and GPIIb/IIIa ($\alpha_{IIb}\beta_3$) with vWf and fibrinogen, as well as perhaps fibronectin, thrombospondin, and/or vitronectin (1–3, 6–18). Some evidence supports a role for GPIa/IIa ($\alpha_2\beta_1$) with collagen (19–21). Other receptor–ligand interactions have been identified, including GPIV (CD36) with collagen (22) and thrombospondin (23), GPIc*/IIa ($\alpha_5\beta_1$) with fibronectin (24), GPIc/IIa ($\alpha_6\beta_1$) (25) and a 67,000-M_r glycoprotein (26) with laminin, and $\alpha_v\beta_3$ with vitronectin, fibrinogen, thrombospondin, fibronectin and vWf (16, 17, 18, 27–29), but their contributions to platelet adhesion are less well defined.

The ability of the adherent platelets to recruit additional layers of platelets via platelet aggregation plays a crucial role in determining the outcome of injury to a normal blood vessel. Thus, although a single layer of platelets may be sufficient to prevent hemorrhage from minimal trauma, it is unlikely to arrest bleeding produced by serious damage to the blood vessel. The platelet recruitment process is equally important in diseased blood vessels. Thus, if the platelet response to rupture of an atherosclerotic plaque is limited to the deposition of a platelet monolayer, there is minimal risk of vaso-occlusion, whereas, if florid platelet thrombus formation occurs, the risks of thromboembolization, vaso-occlusion, and ischemic infarction are dramatically increased.

The processes that determine whether platelet adhesion results in a platelet monolayer or platelet thrombus formation are poorly understood. Factors that are likely to affect the outcome include the biochemical nature of the exposed surface; the rheologic conditions (shear rate, flow patterns, presence of erythrocytes) (30); the biochemical integrity of the platelets (surface receptors, activation pathways, granule contents); and the presence of platelet agonists (e.g., ADP, epinephrine, thrombin, thromboxane A_2, serotonin, and vasopressin), plasma cofactors (fibrinogen, vWf and perhaps fibronectin and vitronectin), and other modifying influences [(PGI_2 (31), nitric oxide (31), ecto-ADPase (32), and cooperative biochemical interactions with erythrocytes (33) and leukocytes (34)].

Since current evidence indicates that platelet–platelet interactions are primarily mediated by the simultaneous binding of the multivalent adhesive glycoproteins fibrinogen and/or vWf to GPIIb/IIIa receptors on two different platelets (35), all of the factors listed above must ultimately operate by affecting the ability of GPIIb/IIIa receptors on an adherent platelet to either capture, or serve as a docking site for, a circulating platelet. To

be more precise, since the GPIIb/IIIa receptor must be activated in order to bind the adhesive glycoproteins with high affinity, and since newly recruited platelets can only bind to the luminal surface of adherent platelets, the presence of physically accessible, activated, GPIIb/IIIa receptors on the luminal surface of adherent platelets is a prerequisite for platelet thrombus formation.

Although it is possible to devise assays involving two separate layers of platelets to assess the presence of activated GPIIb/IIIa receptors on the luminal surface of adherent platelets, such assays are technically cumbersome, and it is difficult to be certain that the second layer of platelets is binding exclusively to the first layer of platelets; moreover, the activation state of the GPIIb/IIIa receptors on the platelets in the second layer is difficult to control, as is their release of adhesive glycoproteins and platelet-activating agents. As a result, we have developed an assay based on the ability of adherent platelets to bind our previously described thromboerythrocytes (36). Thromboerythrocytes are red blood cells with approximately one million peptides containing the RGDF sequence covalently coupled to their surface (36). As we previously reported, the length of the RGD-containing peptide was chosen so that the thromboerythrocytes would bind to activated GPIIb/IIIa receptors on platelets, but not unactivated receptors (36, 37). Thus, the binding of thromboerythrocytes to a lawn of adherent platelets should be a direct reflection of the accessibility of activated GPIIb/IIIa receptors on the luminal surface of adherent platelets.

Methods

Materials. Our preparation of purified, type I collagen from the skin of lathyritic rats was previously described (21). The collagen was stored in 3% acetic acid. Fibrinogen was purified from plasma by sequential precipitations with 0.9 M glycine (5°C) and then 0.4 M glycine. Band I fibrinogen was purified from the supernatant as previously described (38), and then fraction I-3 was purified as per Blomback and Blomback (39). The final material was chromatographed on Sepharose CL-6B (Pharmacia, LKB Biotechnology, Inc., Piscataway, NJ) to remove any high molecular weight soluble fibrin. The fibrinogen had no detectable vWf, vitronectin, or fibronectin as judged by immunoblotting. For some studies, the fibrinogen (1.1–2.5 mg) was radiolabeled with ^{125}I using 25 μg of immobilized Iodogen (Pierce Biochemical Co., Rockford, IL); the specific activity was 500–2,500 cpm/ng. BSA (fraction V) was obtained from Sigma Chemical Co., St. Louis, MO. Purified mAbs 10E5 (anti-GPIIb/IIIa; reference 40), 7E3 (anti-GPIIb/IIIa + $\alpha_v\beta_3$; reference 41), and 6D1 (anti-GPIb; reference 42) have previously been described.

Platelet preparation. Whole blood was anticoagulated with ACD (8.5:1.5) and platelet-rich plasma (PRP) was prepared by centrifugation at 700 *g* for 3.5 min at 22°C. After adding 0.1 vol of additional ACD, the PRP was centrifuged at 2,100 *g* for 10 min at 22°C and the platelet pellet was incubated with $Na_2{}^{51}CrO_4$ for 60 min at 37°C. The platelets were separated from the free ^{51}Cr by gel-filtration on Sepharose-2B (Pharmacia LKB Biotechnology, Inc.) using 0.15 M NaCl, 0.05 M Tris/HCl, 0.5% BSA, 10 mM glucose, pH 7.4. $MgCl_2$, which supports adhesion of gel-filtered platelets to collagen better than calcium (19, 21) and also supports ligand binding to GPIIb/IIIa, was then added to the gel-filtered platelets to achieve a final concentration of 2 mM.

Thromboerythrocytes. Thromboerythrocytes were prepared as previously described by crosslinking the peptide AcCGGRGDF-NH_2 to washed erythrocytes using the heterobifunctional cross-linking agent mal-sac-HNSA (Bachem Bioscience Inc., Philadelphia, PA) (36). For some experiments, thromboerythrocytes were fixed with 0.5% glutaraldehyde (Sigma Chemical Co.) in PBS for 18 h at 4°C and then washed at 22°C and stored at 4°C in 0.15 M NaCl, 0.01 M Tris/HCl, 0.05% azide, 0.1% BSA, pH 7.4 (TSAB). Before use in the assay, thromboerythrocytes were resuspended in 0.15 M NaCl, 0.01 M Tris/HCl, 1% BSA, 10 mM glucose, 5 mM KCl, 2 mM $MgCl_2$, pH 7.4. Control erythrocytes were prepared exactly as thromboerythrocytes, but either the peptide or the cross-linking reagent was omitted.

Thromboerythrocyte adhesion assay. Microtiter plate wells (no. 25805-96; Corning Inc., Corning, NY) were incubated with 100 μl of soluble collagen in acetic acid or fibrinogen in 0.15 M NaCl, 0.01 M Tris/HCl, 0.05% azide, pH 7.4, for 1 h at 37°C and then the wells were blocked with 0.5% BSA in TSA for 60 min at 22°C. The wells were emptied and then 50 μl of the radiolabeled gel-filtered platelets (GFP) at 200,000–400,000 platelets/μl was added and allowed to adhere for 1 h at 22°C. The wells were then washed three times in the same buffer. Control erythrocytes or thromboerythrocytes (50 μl) at concentrations of ~ 510,000/μl (0.5% hematocrit) were then added and allowed to adhere for 1 h at 22°C. After washing three times, the optical density at 405 nm (OD_{405}) of the wells was determined in a microplate reader (Vmax; Molecular Devices Corp., Menlo Park, CA). The OD_{405} of the well containing platelets and control erythrocytes was subtracted from the OD_{405} of the well containing the platelets and thromboerythrocytes, yielding a net OD_{405}, which reflected the density of bound thromboerythrocytes. The contents of each well was then solubilized in 100 μl of 2% SDS and the radioactivity determined in a gamma spectrometer (AutoGamma model 5550; Packard Instrument Co., Meriden, CT).

To establish the relationship between the OD_{405} and the density of fresh and fixed thromboerythrocytes bound to the wells, microtiter plates were coated with collagen at 33 μg/ml and the assay was carried out as above, except that the thromboerythrocyte concentration added to the wells was varied from ~ 10,000–1,200,000/μl in order to obtain a range of thromboerythrocyte densities. The net OD_{405} for each well was determined and then the number of thromboerythrocytes bound per 10,000 μm^2 was determined by direct visual assessment using an inverted microscope (Diaphot-TMD; Nikon, Tokyo, Japan) at a magnification of 100. Fig. 1 shows that there was a nearly linear relationship between the net OD_{405} and the number of erythrocytes bound per 10,000 μm^2; the relationship was the same for unfixed and fixed thromboerythrocytes and for thromboerythrocytes that were either 3 d old or 24 d old. Experiments were performed, therefore, either with fresh or fixed thromboerythrocytes depending on their availability.

To assess the ability of adherent platelets to bind a second layer of platelets as a function of the fibrinogen- and collagen-coating concentrations, the assay was modified and performed in duplicate; one well received radiolabeled platelets and thromboerythrocytes as before, and the other well received a first layer of unlabeled platelets, a subsequent 1-h incubation with platelet-poor plasma (to supply adhesive glycoproteins), and finally a second layer of radiolabeled platelets (1 h). Thus, the radioactivity in the first well reflected the binding of the first layer of platelets, whereas the radioactivity in the second well reflected the binding of the second layer.

Scanning electron microscopy. The adhesion assay was conducted as described above in 96-well polystyrene microtiter plates composed of removable 8-well strips (no. 2580; Costar Corp., Cambridge, MA). After the assays were completed, the wells were rinsed once with PBS (0.15 M NaCl, 0.01 M $NaPO_4$, pH 7.4) and then the cells in the wells were fixed with 100 μl 1.5% glutaraldehyde (LADD-Research Industries, Burlington, VT), 2% paraformaldehyde (Aldrich Chemical Co., Inc., Milwaukee, WI) in 0.1 M cacodylate, pH 7.4, for ~ 60 min at 4°C. The wells were washed twice in 0.1 M cacodylate, pH 7.4, and stored in 0.1 M cacodylate, pH 7.4, at 4°C for up to 3 d. The cells were post-fixed in the wells with 100 μl of freshly prepared 1% osmium tetroxide (Polysciences, Inc., Warrington, PA) in 0.1 M cacodylate, pH 7.4, for 1 h at 22°C. The wells were washed with 0.1 M cacodylate, pH 7.4, and then dehydrated by sequential incubation with water, 30% ethanol, 50% ethanol, 75% ethanol, 95% ethanol, and 100% ethanol. A 1:1 mixture of hexamethyldisilazane (Polysciences, Inc.) and ethanol was then added for 30 min at 22°C, followed by 100% hexamethyldisi-

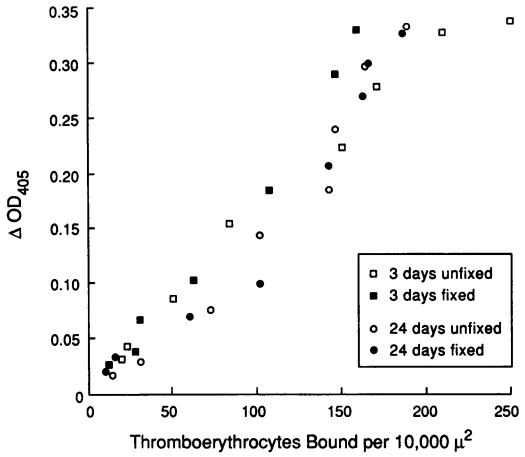

Figure 1. Comparison between net OD_{405} and the number of thromboerythrocytes bound to microtiter wells. The adhesion of gel-filtered, unlabeled platelets to microtiter wells coated with 33 μg/ml purified, type 1, rat skin collagen was performed as described in Methods. Then, either control erythrocytes or thromboerythrocytes were allowed to bind to the adherent platelets; a range of control erythrocyte and thromboerythrocyte concentrations were used to produce different densities of bound control erythrocytes and thromboerythrocytes. The OD_{405} of each well was determined and the net OD_{405} (Δ OD_{405}) was calculated by subtracting the control erythrocyte value from the thromboerythrocyte value. The wells were then visualized with the aid of a microscope and the number of thromboerythrocytes bound per 10,000 μm^2 was determined. There was an essentially linear relationship between net OD_{405} and the density of bound thromboerythrocytes; the values were very similar for unfixed and glutaraldehyde-fixed thromboerythrocytes, and for thromboerythrocytes that were 3 d old or 24 d old.

lazane, first for 1 h at 22°C and then for 2 h at 22°C. The plate was placed in a sealed dessicator with calcium sulfate dessicant (Drierite; W. A. Hammond Drierite Co., Xenia, OH), but no vacuum, overnight to evaporate the hexamethyldisilazane. The individual wells were then separated from the strip. The bottom of each well, containing the adherent cells, was then separated from the walls by briskly cutting just above the bottom with a pet nail cutter (Miller's Forge, Plano, TX). The bases of the cut wells were then attached to 1 cm aluminum holders (no. 76510; Electron Microscopy Sciences, Ft. Washington, PA) using double-sided tape and the samples were coated with gold–palladium, using an automated device (Hummer VI-A-Sputtering System; EM Corp., Chestnut Hill, MA; 70 mtorr, 10 mAmps, 4 min). Colloidal silver (Electron Microscopy Sciences) was then painted on the side of the specimen to make contact with the holders and allowed to dry. The specimens were visualized in a scanning electron microscope (model JSM-5300; JEOL, Tokyo, Japan) at 10 KV with tilt angles of 40–52°. Photographs were taken with an instant camera (Polaroid, Type 52 film, ASA 400).

Immunogold scanning electron microscopy. Platelet adhesion to protein-coated microtiter wells was conducted as before, but with unlabeled GFP. After washing, 100 μl of buffer containing 1 μg/ml of antibody 10E5 or 6D1 was added and incubated for 30 minutes at 22°C. After washing three times, 100 μl of 0.15% formaldehyde (Electron Microscopy Sciences), 0.004% glutaraldehyde (Sigma Chemical Co.) was added and incubated for 1 h at 22°C. After three additional washes, gold-labeled, goat anti-mouse IgG (Fc; 125 μl; 30 nm; Auroprobe EM GAM IgG G30; Amersham Corp., Arlington Heights, IL) diluted 1/10 in buffer was added and incubated for an additional ~ 1 hour at 22°C. The wells were then further fixed with 1.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate, pH 7.4, for 1 h at 4°C and reacted with 1% osmium tetroxide in 0.1 M cacodylate at 22°C as above. Samples were further processed as above for scanning electron microscopy. To enhance the detection of the gold particles, the specimens were viewed directly from above and both secondary emitted images (SEI)[1] and backscatter emitted images (BEI) were obtained.

Results

Platelet adhesion and thromboerythrocyte binding. The results of 18 separate experiments conducted on platelets obtained from 8 separate donors are shown in Fig. 2. As we previously reported, platelets readily adhere to microtiter wells coated with purified soluble, type I, rat skin collagen at 33 μg/ml, and these adherent platelets are able to bind a layer of thromboerythrocytes (36). The surface density of both platelets and thromboerythrocytes was similar when the wells were coated with 3.3 μg/ml of collagen rather than 33 μg/ml. In sharp contrast, over a similar range of fibrinogen concentrations used for coating the wells, both the ability of platelets to adhere to the wells and the ability of the adherent platelets to bind thromboerythrocytes differed dramatically as a function of the fibrinogen concentration. Platelet adhesion increased as the concentration of fibrinogen used to coat the wells increased from ~ 1.5–11 μg/ml; above this concentration of fibrinogen there was little or no additional increase in platelet adhesion, even at 33 and 100 μg/ml (Fig. 2). At the higher fibrinogen concentrations, the density of adherent platelets was very similar to that obtained with collagen, suggesting that this represents a near maximal density for a monolayer. Thromboerythrocyte binding to the platelets adherent to fibrinogen also differed as a function of the fibrinogen concentration used to coat the wells, but the pattern did not follow that observed with platelets. The number of thromboerythrocytes bound per well increased as the fibrinogen-coating concentration increased from 1.5–4.6 μg/ml, plateaued in the range between 4.6–6.2 μg/ml, and then declined at higher fibrinogen-coating concentrations. At the highest fibrinogen-coating concentrations, the number of bound thromboerythrocytes was very low, averaging less than 5% of the value obtained at 4.6 μg/ml. This pattern of thromboerythrocyte binding was reproducible from experiment to experiment and with different platelet donors, although there were minor variations in the fibrinogen concentration producing the peak value of thromboerythrocyte binding: In the 18 separate experiments, peak thromboerythrocyte binding occurred at 6.2 μg/ml fibrinogen in 8 experiments, at 4.6 μg/ml in 7 experiments, and at 8.3 μg/ml, 3.5 μg/ml, and 2.6 μg/ml in 1 experiment each. Although the possibility was considered that thromboerythrocyte binding was inhibited at the higher fibrinogen-coating concentrations because of the increased density of adherent platelets, this hypothesis was considered unlikely because platelets adherent to collagen at a similarly high density were very effective in binding thromboerythrocytes.

Inhibition of thromboerythrocyte binding to adherent platelets by mAbs. We previously demonstrated that mAb 10E5, directed against the GPIIb/IIIa receptor, and an RGD-containing soluble peptide could inhibit the binding of thromboerythrocytes to platelets adherent to collagen-coated microtiter wells (36). This supported the hypothesis that thromboerythrocyte binding is specifically mediated by the interaction

1. *Abbreviations used in this paper:* BEI, backscatter emitted images; SEI, secondary emitted images.

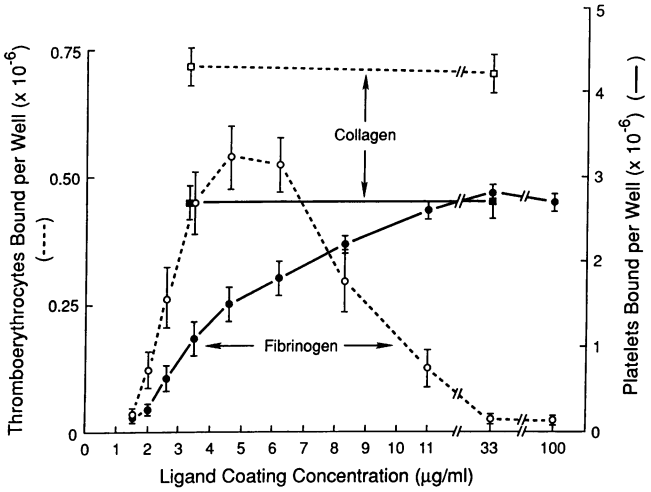

Figure 2. Platelet and thromboerythrocyte binding as a function of the concentration of the ligand used to coat the microtiter wells. The adhesion assay was performed as described in Methods using wells coated with varying concentrations of collagen or fibrinogen. Thromboerythrocyte binding was assessed by measuring the net OD_{405} and the adhesion of ^{51}Cr-labeled platelets was assessed by the radioactivity in the wells. For wells coated with collagen at 3.3 or 33 μg/ml, both platelet adhesion and thromboerythrocyte binding were similar. Over a similar range of fibrinogen-coating concentrations, platelet adhesion increased with higher fibrinogen-coating concentrations, plateauing at ~ 11 μg/ml. Thromboerythrocyte binding demonstrated a biphasic pattern, increasing as the fibrinogen-coating concentration increased to 4.6–6.2 μg/ml and then decreasing almost to baseline at higher concentrations. The data shown are from 18 separate experiments conducted with platelets from 8 separate donors. The mean and SEM are depicted for each data point.

between the RGD-containing peptides on the surface of the thromboerythrocytes and the luminal GPIIb/IIIa receptors on the adherent platelets. Our new assay permits us to quantify these observations and to assess whether similar results are obtained with platelets adherent to fibrinogen. As shown in Fig. 3, antibody 6D1, directed against the GPIb receptor, had no effect on thromboerythrocyte binding. In contrast, adding 50 μg/ml antibody 10E5, directed against GPIIb/IIIa, to platelets after they adhered to the microtiter wells caused profound inhibition of subsequent thromboerythrocyte binding to platelets adherent to either fibrinogen (84% inhibition at 4.6 μg/ml) or collagen (76% inhibition at 33 μg/ml). Antibody 7E3, directed against GPIIb/IIIa + $\alpha_v\beta_3$, produced virtually complete inhibition of thromboerythrocyte binding to platelets adherent to either adhesive protein (> 99% in both cases). The inhibition caused by 10E5 and 7E3 was not a result of detaching platelets from the surface, since the densities of adherent platelets at the concentrations indicated in Fig. 3 were very similar between the control and antibody-treated wells (data not shown).

Comparison of thromboerythrocytes bound per platelet and density of fibrinogen on the microtiter plates. Using the data in Fig. 2, an index of the ability of an adherent platelet to bind thromboerythrocytes was obtained by dividing the number of thromboerythrocytes bound per well by the number of platelets bound per well. The resulting values of thromboerythrocytes bound per platelet are shown in Fig. 4 as a function of the protein coating concentrations of collagen and fibrinogen. The values for platelets adherent to collagen at either 3.3 or 33 μg/ml were virtually identical (~ 0.3 for both). In sharp contrast, the thromboerythrocyte binding per platelet values varied dramatically as a function of the fibrinogen concentration. A maximal value was observed at 2.0 μg/ml; using higher fibrinogen-coating concentrations resulted in a progressive decrease in the ability of the adherent platelets to bind thromboerythrocytes, with the values dropping nearly to zero at the highest concentrations.

The dependence of thromboerythrocyte binding on the fibrinogen-coating concentration was further explored by directly assessing the number of molecules of fibrinogen bound per well, and then calculating the number of fibrinogen molecules that would be expected to be under a fully spread platelet, estimated to be ~ 25 μm^2 from our scanning electron micrographs (see below). Studies with radiolabeled fibrinogen demonstrated that the percentage of added fibrinogen that bound per well decreased as the concentration of added fibrinogen increased, going from ~ 46% at 1–22 μg/ml to ~ 32% at 33 μg/ml, and ~ 15% at 100 μg/ml. Analysis of the bound fibrinogen as a function of the surface area coated, led to the determination that at the 100 μg/ml fibrinogen-coating concentration, more than 700,000 fibrinogen molecules would be expected to be underneath a fully spread platelet, whereas at 2 μg/ml, only ~ 20,000 fibrinogen molecules would be beneath a spread platelet (Fig. 4). Since the number of surface GPIIb/IIIa receptors per platelet is probably ~ 40,000–80,000 (40, 43, 44), with perhaps another 20,000–40,000 receptors in internal stores (41, 45), it is interesting to note that the marked reduction in the ability of adherent platelets to support thromboerythrocyte binding occurs as the number of fibrinogen molecules under a spread platelet exceeds the number of GPIIb/IIIa receptors (Fig. 4).

Assessment of the ability of adherent platelets to bind a second layer of platelets. As shown in the representative experi-

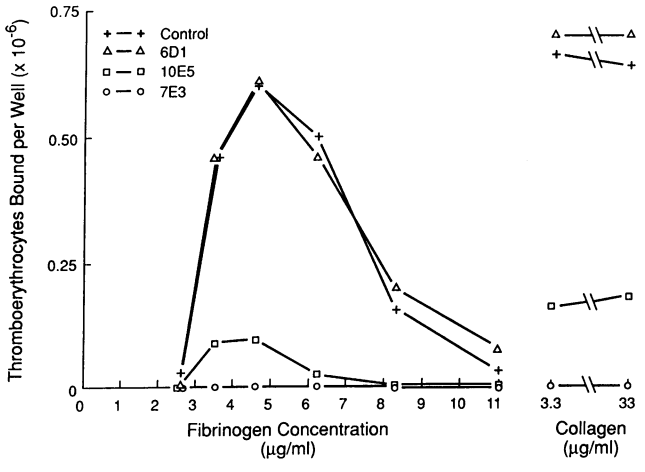

Figure 3. Effect of mAbs on thromboerythrocyte binding to adherent platelets. The adhesion assay was conducted as described in Methods except that mAbs 6D1 (anti-GPIb), 10E5 (anti-GPIIb/IIIa), or 7E3 (anti-GPIIb/IIIa + $\alpha_v\beta_3$) were incubated with the platelets after platelet adhesion and throughout the thromboerythrocyte binding step. In this representative experiment, the control was similar to that depicted in Fig. 2 and antibody 6D1 had no effect on thromboerythrocyte binding. Antibody 10E5 caused a marked, but incomplete inhibition of thromboerythrocyte binding, whereas antibody 7E3 produced virtually complete inhibition.

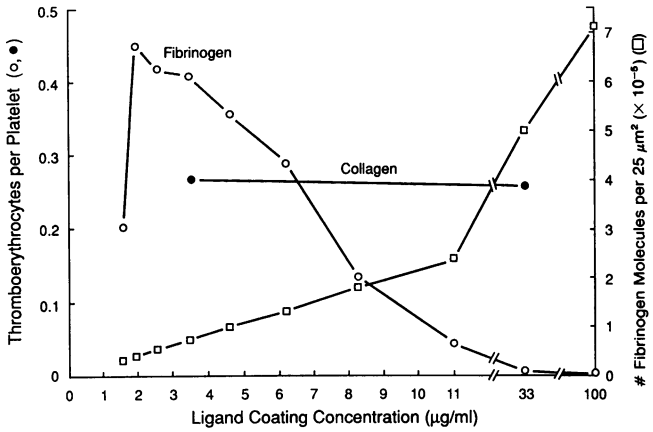

Figure 4. Thromboerythrocyte binding per platelet as a function of fibrinogen- and collagen-coating concentrations; fibrinogen molecules bound per 25 μm^2 of microtiter well area as a function of fibrinogen-coating concentrations. The data from Fig. 2 were used to calculate the number of thromboerythrocytes bound per platelet for the different collagen- and fibrinogen-coating concentrations. In three separate experiments, ^{125}I-labeled fibrinogen was used to determine the amount of fibrinogen bound per well at the different fibrinogen-coating concentrations; from these data the number of fibrinogen molecules bound per 25 μm^2, the area estimated to be under a fully spread platelet, was calculated. For collagen, the number of thromboerythrocytes bound per platelet was similar at collagen-coating concentrations of 3.3 and 33 μg/ml, whereas the number of thromboerythrocytes bound per platelet was highest at a fibrinogen-coating concentration of ~ 2 μg/ml and showed a steady downward progression at higher fibrinogen-coating concentrations. The number of fibrinogen molecules per 25 μm^2 was ~ 700,000 at a fibrinogen-coating concentration of 100 μg/ml, 200,000 at ~ 9 μg/ml, 100,000 at ~ 4 μg/ml, and ~ 20,000 at 2 μg/ml.

ment depicted in Fig. 5, the adhesion of the first layer of platelets and the binding of the thromboerythrocytes followed the same pattern as shown in Fig. 2. The binding of the second layer of platelets to the adherent platelets incubated with platelet-poor plasma, followed a pattern similar to that observed with the thromboerythrocytes, with peak binding to platelets adherent to wells coated with fibrinogen at 4.6 μg/ml and a decrease in binding at the higher fibrinogen-coating concentrations. The decrease in the binding of the second layer of platelets at the higher fibrinogen-coating concentrations was not, however, as dramatic as the decrease in thromboerythrocyte binding in this experiment, although it did approximate the mean values depicted in Fig. 2. The binding of the second layer of platelets to platelets adherent to collagen also followed the same pattern observed with thromboerythrocytes, with high density binding to platelets adherent to wells coated with either 3.3 or 33 μg/ml. In addition, as with the thromboerythrocytes, the density of binding of the second layer of platelets was higher when collagen was the substrate than when fibrinogen was the substrate. When purified fibrinogen at concentrations between 150–500 μg/ml was substituted for platelet-poor plasma as the solution used to interact with the already adherent platelets, the same biphasic pattern of second layer platelet binding was also observed (data not shown).

Scanning electron microscopy. The morphology of platelets adherent to collagen at 1 μg/ml and 33 μg/ml was essentially the same, with extensive membrane ruffling and filopodia formation, and extension of the filopodia from the central region of the platelet both along the surface of the well and up from the surface into the equivalent of the luminal space (Fig. 6 *A*). Thromboerythrocytes were readily seen binding to the platelets adherent to collagen, and binding appeared to be primarily due to attachment of filopodia extending up from the surface of the well onto the surface of the thromboerythrocytes. At a low fibrinogen-coating concentration (1 μg/ml), the morphology of adherent platelets was similar to that observed with collagen at both low and high coating concentrations (Fig. 6 *B*). There were, however, focal areas where there appeared to be more extensive spreading of the platelets. Binding of thromboerythrocytes to the platelets adherent to the wells coated with the low concentrations of fibrinogen was similar to that with platelets adherent to collagen. In marked contrast, the morphology of platelets adherent to wells coated with a high fibrinogen concentration (33 μg/ml) was dramatically different, with very few filopodia extending up from the surface of the well and extensive, flat spreading of the platelets. The region of the platelet containing the organelles protruded as a mound, giving the entire platelet a fried egg appearance. When fully spread, platelets occupied areas of ~ 5 μm × 5 μm, or ~ 25 μm^2. It was extremely difficult to identify any thromboerythrocytes bound to the platelets adherent to the wells coated with the high fibrinogen concentrations, and when they were observed, a platelet filopodium extending up from the luminal surface was usually seen to be attached to the thromboerythrocyte. Thus, the di-

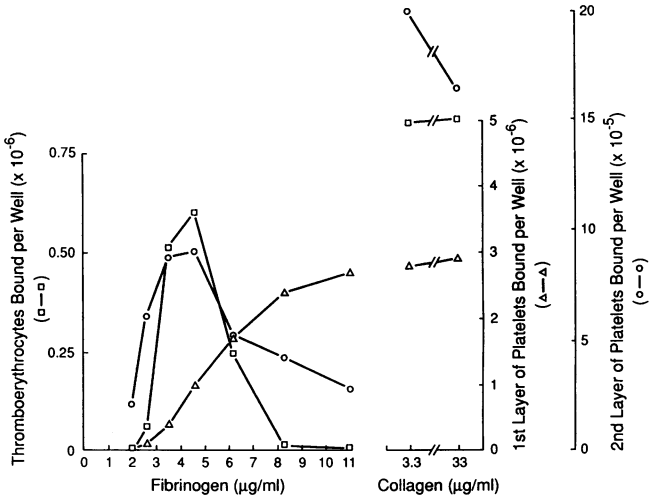

Figure 5. Platelet adhesion, thromboerythrocyte binding, and binding of a second layer of platelets as a function of ligand-coating concentration. The adhesion assay was performed in duplicate: one plate was used to assess platelet adhesion and thromboerythrocyte binding as described in Methods; in the other plate, the assay was modified so that unlabeled platelets were used in the initial adhesion step, plasma was then incubated with the adherent first layer of platelets, and finally a second layer of ^{51}Cr-labeled platelets was allowed to bind to the first layer of adherent platelets. In this experiment, the adhesion of the first layer of platelets and thromboerythrocyte binding were similar to that observed in Fig. 2, although the decline in thromboerythrocyte binding was more pronounced as the fibrinogen-coating concentration increased. The binding of the second layer of platelets, was similar in pattern to that of the thromboerythrocytes, with higher values for collagen-coated surfaces than fibrinogen-coated surfaces, and a biphasic pattern for binding to platelets adherent to fibrinogen-coated surfaces.

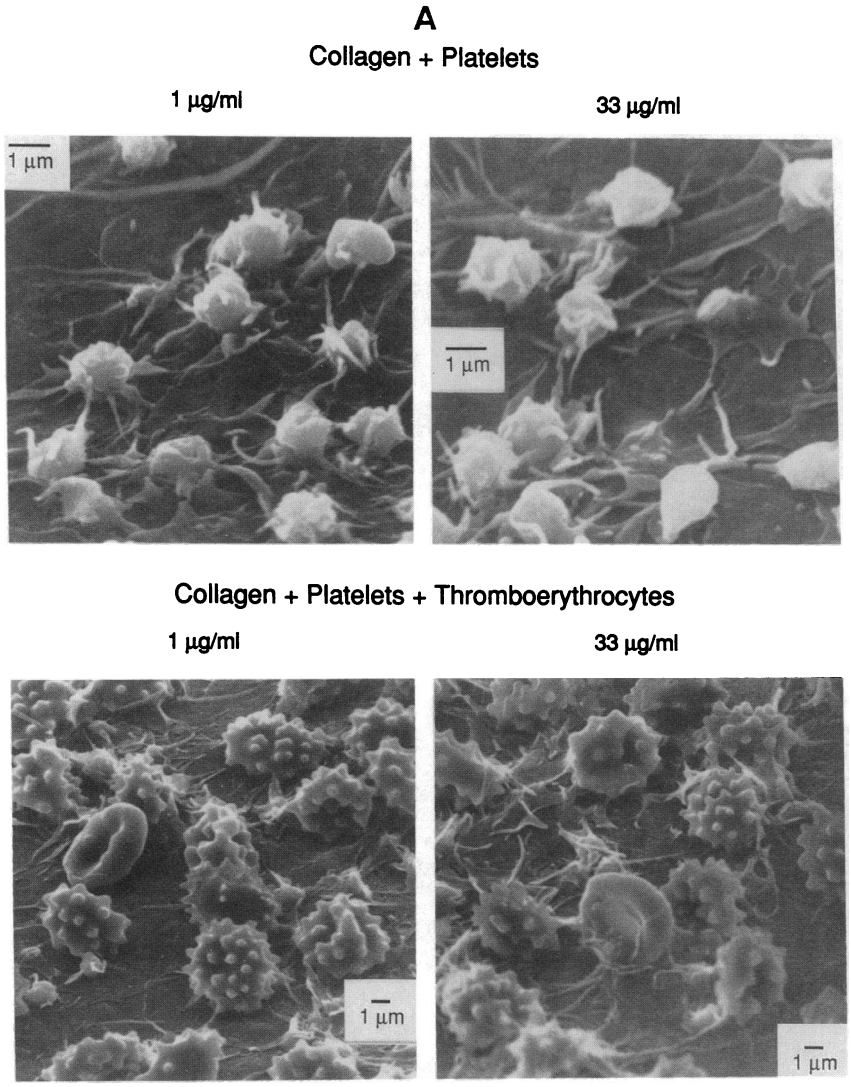

Figure 6. Scanning electron micrographs of platelets and thromboerythrocytes adherent to collagen (*A*) and fibrinogen (*B*). In *A* the morphology of adherent platelets (*upper panels*) was similar at collagen coating concentrations of 1 μg/ml and 33 μg/ml, with extensive ruffling and filopodia formation. Filopodia extended both downward onto the collagen surface and upward into the equivalent of the luminal space. Filopodia participated in binding the thromboerythrocytes to the platelet lawn (*lower panels*). In *B* platelets adherent to wells coated with the low concentration of fibrinogen (1 μg/ml) had a morphology similar to that of platelets adherent to collagen at both 1 μg/ml and 33 μg/ml, although there were some areas where the platelets spread more extensively (*upper panel*). Thromboerythrocyte binding to platelets adherent to the well coated with 1 μg/ml of fibrinogen (*lower panel*) was similar to that seen with collagen-coated wells. In contrast, platelets adherent to wells coated with 33 μg/ml of fibrinogen had a distinctly different morphology, with extensive spreading and flattening, and very few filopodia extending up from the platelet. It was very difficult to identify any thromboerythrocytes bound to platelets adherent to wells coated with 33 μg/ml of fibrinogen (*lower panel*). The few thromboerythrocytes that were identified appeared to be bound to the few platelets that still extended filopodia.

minished ability of platelets adherent to wells coated with high concentrations of fibrinogen to bind thromboerythrocytes correlated with morphological changes characterized by extensive platelet spreading along the surface of the well and the absence of filopodia extending up from the surface of the well.

Immunoelectron microscopy. To directly assess the presence of GPIIb/IIIa receptors on the surface of adherent platelets, immunoelectron microscopy with antibodies to GPIIb/IIIa and GPIb was performed. Platelets adherent to collagen (33 μg/ml) and low density fibrinogen (4 μg/ml) bound antibody 10E5 (anti–GPIIb/IIIa) readily, with the filopodia staining most intensely (Fig. 7 *A*). Platelets that had attached and spread on high density fibrinogen (100 μg/ml), however, showed very little binding of 10E5 to the spread surface of the platelets and virtually all of the binding was confined to the few regions where small ruffles and filopodia could be identified (Fig. 7 *B*). To assess whether the apparent absence of GPIIb/IIIa antigen from the spread region of the platelet was specific, the binding of antibody 6D1 (anti–GPIb) was also tested. It bound extensively to the spread surface of the platelets adherent to the high density fibrinogen, indicating that loss of GPIIb/IIIa antigen was not part of a nonspecific receptor reaction to spreading on high density fibrinogen (Fig. 7 *B*). It also demonstrated that spreading on the high density fibrinogen did not produce a generalized loss of accessibility of receptors to mAbs. The experiments in Fig. 7 were performed by incubating the adherent platelets with the mAbs and then gently fixing the platelets before adding the gold-labeled second antibody. Similar results were observed when the gentle fixation was performed before the first antibody was added (data not shown).

Discussion

Although the accessibility of activated, GPIIb/IIIa receptors on the surface of adherent platelets plays an important role in hemostasis and thrombosis, technical complexity has made it a difficult phenomenon to study. Savage et al. studied the adhesion of platelets in PRP prepared from blood anticoagulated with the thrombin inhibitor D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone dihydrochloride (PPACK) to microtiter wells coated with fibrinogen or vWf at a single concentration (50 μg/ml; reference 46). The binding of 2 different mAbs to GPIIb/IIIa was measured: LJ-P4, an IgG antibody that reacts independently of GPIIb/IIIa activation and PAC1, an IgM mAb specific for the activated form(s) of the GPIIb/IIIa receptor (47). The binding of LJ-P4 was taken as an indica-

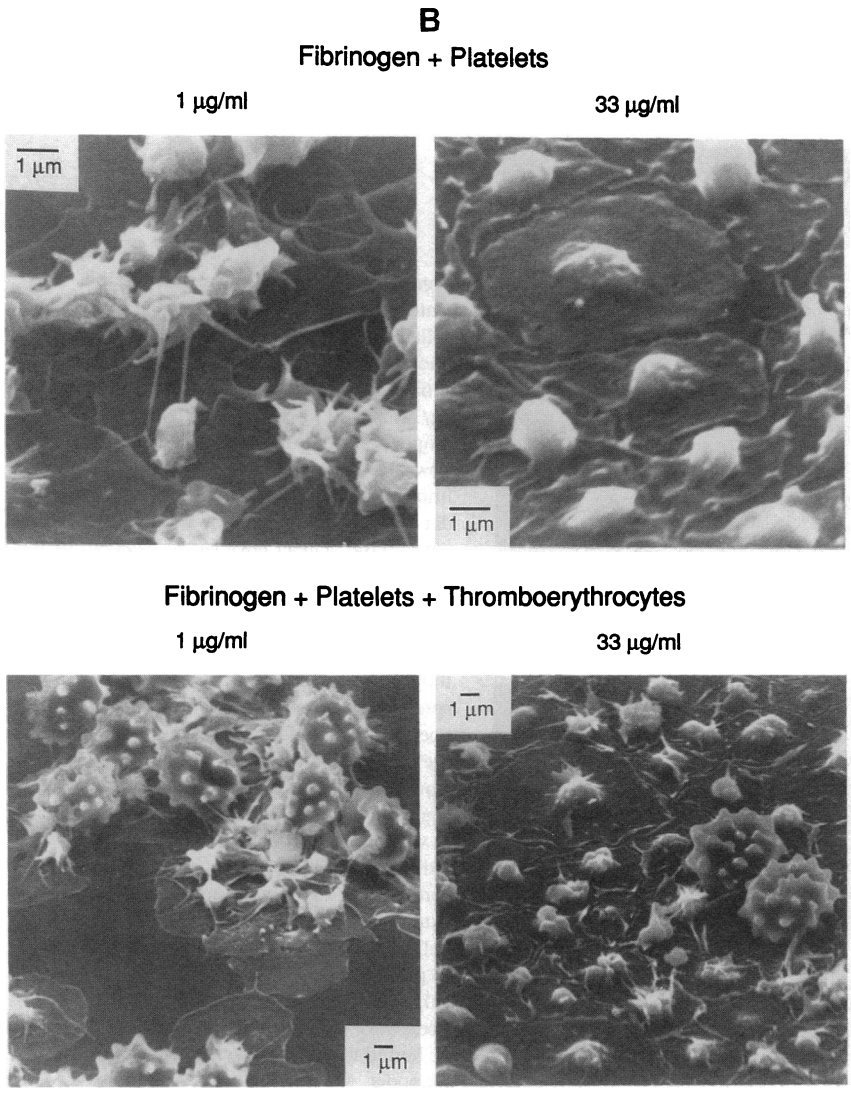

Figure 6. (Continued)

tion of total platelet adhesion and the binding of PAC1 represented activation of surface GPIIb/IIIa receptors. With untreated PRP, approximately 9.5 times as many LJ-P4 molecules bound per well as did PAC1 molecules, whereas with PRP treated with inhibitors of platelet activation, the ratio was ~ 32. These data were interpreted as indicating that platelet adhesion to fibrinogen in the absence of inhibitors resulted in activation of unoccupied GPIIb/IIIa receptors. Although the ratio of LJ-P4/PAC1 binding to platelets in suspension was not reported, if LJ-P4 binding is similar to the binding of other mAbs to GPIIb/IIIa (40, 41, 45), ~ 40,000 molecules will bind per platelet, whereas ~ 12,500 PAC1 molecules will bind to a platelet fully activated with ADP or epinephrine; the values for both antibodies will increase by approximately twofold if platelets are activated by thrombin (45, 47). Thus, one would expect a ratio of LJ-P4/PAC1 binding of ~ 3.2 if the platelets are fully activated. The higher (9.5:1) ratio of observed LJ-P4/PAC1 binding to adherent platelets may represent a lack of maximal receptor activation, occupancy of GPIIb/IIIa receptors by fibrinogen, or perhaps loss of GPIIb/IIIa receptors from the luminal to the adherent platelet surface, where they may be able to react with LJ-P4 but not PAC1. Since only a single concentration of fibrinogen was used, the results of that study cannot be directly compared to the current study. Interpretation of the results of the antibody binding studies is also complicated by uncertainties about whether GPIIb/IIIa receptors that are directly involved in adhesion are equally reactive with, or accessible to, LJ-P4, and whether PAC1 can displace immobilized ligands from GPIIb/IIIa, and if so, over what time frame. Finally, it is conceivable that a GPIIb/IIIa molecule may be activated and accessible to a soluble PAC1 molecule, but may not be able to interact with an adhesive ligand that is simultaneously attached to another platelet. Haimovich et al. (48) also studied PAC1 binding to adherent platelets and compared the results using surfaces coated with either fibrinogen (100 μg/ml) or collagen (50 μg/ml bovine type I calfskin). It is of interest that approximately four times as much PAC1 bound per platelet when the platelets were adherent to collagen as when they adhered to fibrinogen, a result that appears to be consistent with our findings.

In an attempt to define better the functional capacity of activated GPIIb/IIIa receptors on the luminal surface of adherent platelets, and to avoid the technical and interpretative problems associated with assays based on the binding of two

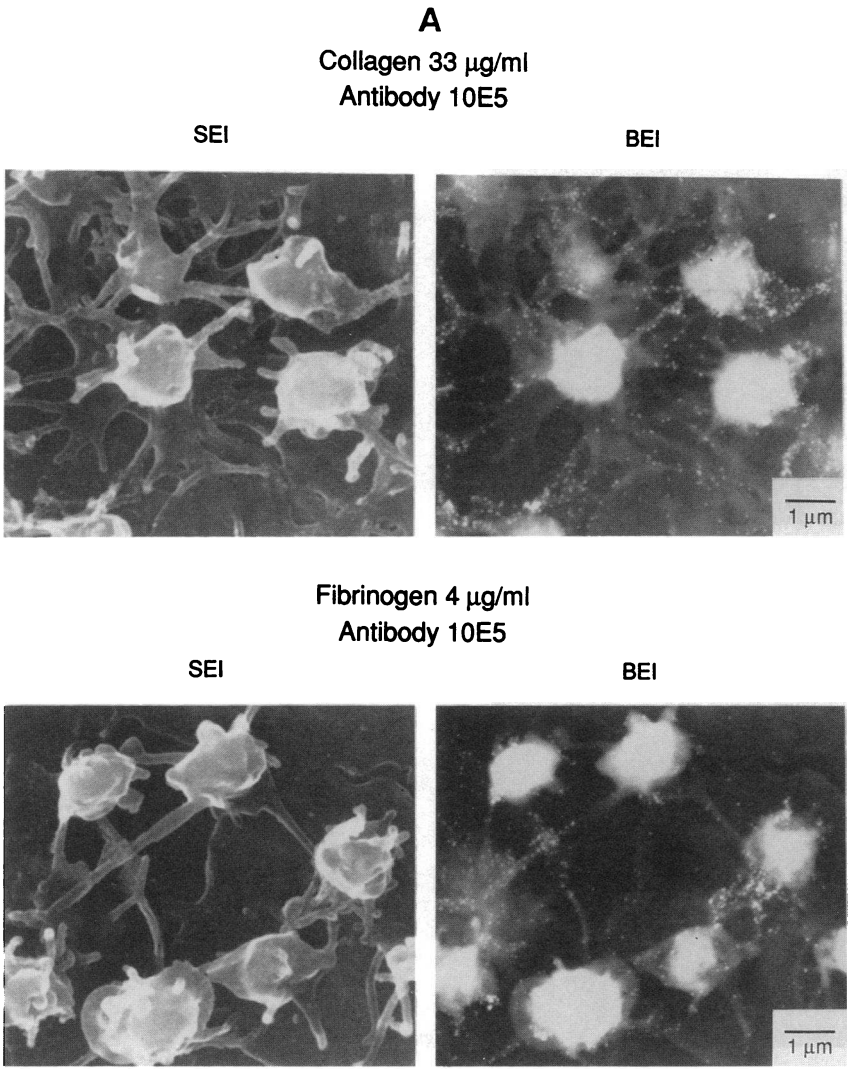

Figure 7. Scanning electron micrographs displaying SEI and BEI of immunogold-labeled platelets adherent to collagen and fibrinogen. After the adhesion assay was performed, the platelets were incubated with mAb 10E5 to identify GPIIb/IIIa receptors and 6D1 to identify GPIb receptors. The antibody-treated platelets were gently fixed (0.15% formaldehyde, 0.004% glutaraldehyde for 1 h at 22°C) and the mAbs were visualized with gold-labeled antibody to mouse IgG. (*A*) When platelets were adherent to collagen at 33 μg/ml or low density fibrinogen (4 μg/ml), GPIIb/IIIa receptors were identified primarily on the filopodia, but with some reactivity on the body of the platelets. (*B*) When platelets were adherent to high density fibrinogen (100 μg/ml), their spread surfaces were nearly devoid of GPIIb/IIIa receptors as judged by 10E5 binding (*upper panel*). Spreading did not lead to nonspecific loss of all platelet surface receptors, however, since antibody 6D1 (anti-GPIb) reacted strongly with the surface of the spread platelet (*lower panel*).

separate layers of platelets or the binding of mAbs, we have developed an assay that relies on the ability of thromboerythrocytes (erythrocytes that contain a high density of RGD-containing peptides of selected length on their surface [36]), to bind to an adherent layer of platelets. We chose gel-filtered platelets to minimize any confounding effects of plasma proteins binding to either the adhesive protein coatings on the wells or to the GPIIb/IIIa receptors. Similarly, washed thromboerythrocytes in buffer were used.

Results from the thromboerythrocyte assay indicate that platelet adhesion to collagen makes activated GPIIb/IIIa molecules readily available even when using a tenfold range of collagen-coating concentrations. This finding is in accord with, and helps to explain, the well-recognized thrombogenicity of surfaces coated with collagen, and the increased platelet deposition that occurs when deep blood vessel injury exposes collagen (49).

Our most striking finding was that thromboerythrocyte binding showed a biphasic pattern as a function of fibrinogen coating concentration; there was an increase in thromboerythrocyte binding at fibrinogen coating concentrations up to ~ 4–6 μg/ml and then a decrease at higher fibrinogen concentrations. When expressed as thromboerythrocyte binding per platelet, the peak was at 2 μg/ml of fibrinogen, with a progressive decline to < 5% of the peak value at higher fibrinogen coating concentrations. A similar pattern of binding was observed when plasma and a second layer of platelets was substituted for the thromboerythrocytes. This latter assay is technically more cumbersome because of the need to perform parallel assays to calculate the binding of the first and second layers of platelets separately; it also requires that two different platelet preparations (labeled and unlabeled) behave identically. In addition, the potential for the added plasma proteins, and perhaps proteins variably released from the second layer of platelets, to bind to the protein coating the well adds another variable, leading to concerns about whether the second layer of platelets is truly binding just to the first layer rather than to new proteins adsorbed on the well. Moreover, the two-layer assay is critically dependent on the activation state of the GPIIb/IIIa receptors on the second layer of platelets, and this is difficult to control. Thus, although it is comforting that the results from the thromboerythrocyte and two platelet layer assays are similar, it may well be that the thromboerythrocyte assay is a more reliable indicator of luminal GPIIb/IIIa receptor activity.

One possible explanation for the decrease in thromboerythrocyte and platelet binding at higher fibrinogen coating

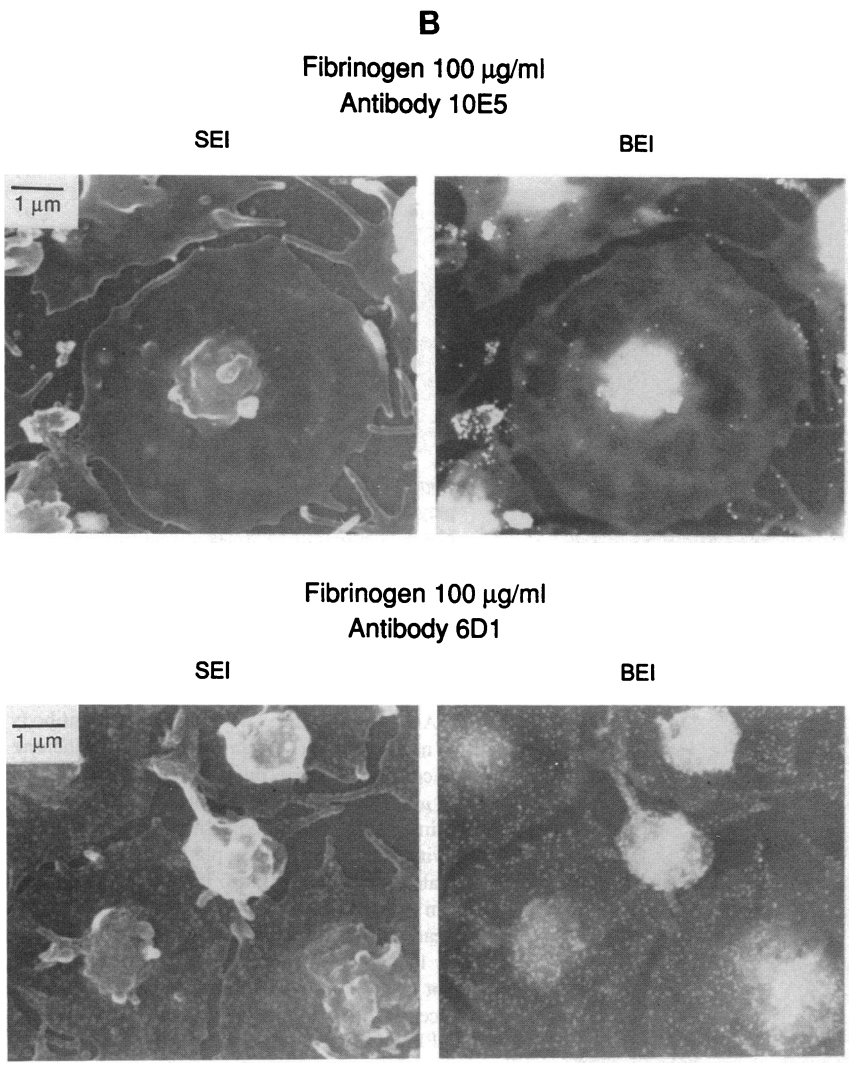

Figure 7. (Continued)

concentrations is that since fibrinogen is itself a ligand for GPIIb/IIIa, a high density of fibrinogen on the well could strip the luminal surface of GPIIb/IIIa receptors. Such a hypothesis requires that GPIIb/IIIa receptors are freely mobile on the platelet surface. Inferences derived from studies of GPIIb/IIIa cycling to the α granule pool and back (50, 51), and movement of GPIIb/IIIa molecules under the influence of certain stimuli (52–55), appear to support the ability of GPIIb/IIIa receptors to move under some circumstances, but no direct observations of GPIIb/IIIa movement after platelet adhesion to fibrinogen have been made.

The hypothesis also requires that there be sufficient fibrinogen beneath a platelet to engage all of the GPIIb/IIIa receptors available. To judge this better, we used radiolabeled fibrinogen to calculate the number of fibrinogen molecules that would be under a fully spread platelet ($\sim 25\ \mu m^2$). At the highest fibrinogen concentrations, the number of fibrinogen molecules ($\sim$ 700,000) greatly exceeds the number of GPIIb/IIIa molecules, i.e., $\sim$ 40,000–80,000 if only the surface receptors are involved, and $\sim$ 60,000–120,000 if both the surface and internal pools are involved (40, 41, 45). At the fibrinogen coating concentration that results in the maximal ratio of thrombo-erythrocyte binding per platelet ($\sim 2\ \mu g/ml$), only $\sim$ 20,000 fibrinogen molecules are beneath a spread platelet. Since it is not certain how many of the six potential sites each fibrinogen molecule has for interaction with GPIIb/IIIa receptors (four RGD-containing regions and two gamma chain carboxy-terminal regions) are actually engaged, precise stoichiometric arguments are difficult to make. Moreover, it is possible that the conformation, and thus the accessibility, of the immobilized fibrinogen molecules differ significantly as a function of the coating concentrations, making speculation even more difficult. Nonetheless, our fibrinogen density data are at least consistent with a hypothesis involving movement of surface GPIIb/IIIa receptors to the abluminal surface. It also remains possible, however, that adhesion of platelets to high density fibrinogen initiates intracellular changes that result in GPIIb/IIIa internalization rather than abluminal trapping.

Our findings with platelets adherent to fibrinogen are analogous to those of Michl et al. (56) who studied macrophage binding to glass surfaces coated with antigen-IgG complexes. As the density of antigen-IgG complexes used to coat the glass increased, the number of Fc receptors remaining on the luminal surface of the cell decreased as judged both by antibody binding and by a functional assay utilizing antibody-coated sheep erythrocytes. Quantitative analysis indicated maximal loss of luminal surface receptors when the number of immobilized IgG molecules was 20-fold greater than the total number

of Fc receptors on the macrophages. They went on to show that loss of luminal receptors was due to lateral diffusion of receptors in the membrane, a process that was sensitive to temperature, but not cytoskeletal-disrupting agents, metabolic inhibitors, or even fixation with low doses of formaldehyde (57). The diffusion process was extremely rapid, being nearly complete in 2 min at 37°C.

We found a remarkable difference in the morphology of the platelets adherent to the high density fibrinogen as compared to the low density fibrinogen. With the low density fibrinogen, the platelets had extensive filopodia and ruffling extending both onto the surface of the well and extending up from the surface. This morphology was very similar to that observed with platelets adherent to collagen. In contrast, the platelets adherent to high density fibrinogen were spread flat, with the rounded protrusion of the organelles in the center giving a fried egg appearance. We speculate that the larger number of GPIIb/IIIa receptors that are able to tack down the platelets on the high density fibrinogen contribute to the appearance, with the possibility that other spreading mechanisms are also triggered as a result of the interactions. Our direct assessment of adherent platelets for GPIIb/IIIa antigen with mAbs and immunogold reagents demonstrated concentration of GPIIb/IIIa receptors on filopodia and ruffled areas of platelets adherent to collagen or low density fibrinogen, and depletion of GPIIb/IIIa on the flattened surfaces of the platelets adherent to the high density fibrinogen. The latter depletion was specific for GPIIb/IIIa, since an antibody to GPIb showed strong reactivity over the flattened surfaces of the platelets adherent to high density fibrinogen.

These morphologic and immunologic studies add another dimension to our analysis of the paradoxical loss of thromboerythrocyte binding to platelets adherent to high density fibrinogen. Since we observed that platelets bind thromboerythrocytes via their filopodia, it is possible that even if surface GPIIb/IIIa receptors are retained on the luminal surface of the adherent platelets, the absence of filopodia and ruffling limits thromboerythrocyte, and perhaps platelet, binding.

In summary, in this study we have begun a systematic analysis of the biochemical and morphological changes in platelets after adhesion to fibrinogen and collagen. These changes affect their ability to make activated GPIIb/IIIa receptors accessible on the luminal surface in a way that allows for the recruitment of additional platelets. We expect that these data will provide insights into the differences in thrombogenicity between surfaces and we hope that they will provide information that can be exploited to purposefully passivate platelet-reactive artificial surfaces. They also provide a potential hypothesis to explain the loss of thrombogenicity that occurs with time after blood vessel injury by angioplasty or other insults (58–60), since Hatton et al. (61) reported that in rabbits fibrinogen saturates the denuded aortic surface 10 min after balloon injury deendothelialization at a level of 13 pmol/cm^2, which corresponds to $\sim 2 \times 10^6$ molecules/25 μm^2.

Acknowledgments

We would like to thank Shirley Murray for outstanding secretarial assistance.

This work was supported by grant 19278 from the National Heart, Lung and Blood Institute.

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