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Research Article

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Thrombospondin–Platelet Interactions

Role of Divalent Cations, Wall Shear Rate, and Platelet Membrane Glycoproteins

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Abstract

The role of thrombospondin, a multifunctional matrix glycoprotein, in platelet adhesion is controversial: both adhesive and antiadhesive properties have been attributed to this molecule. Because shear flow has a significant influence on platelet adhesion, we have assessed thrombospondin–platelet interactions both under static and flow conditions. The capacity of thrombospondin to support platelet adhesion depended upon its conformation. In a Ca^{2+}-depleted conformation, such as in citrated plasma, thrombospondin was nonadhesive or antiadhesive as it inhibited platelet adhesion to fibrinogen, fibronectin, laminin, and von Willebrand factor by 30–70%. In a Ca^{2+}-replete conformation, however, thrombospondin effectively supported platelet adhesion. Shear rate influenced this adhesion; percent surface coverage on thrombospondin increased from 5.4±0.3 at 0 s^{-1} to 41.5±6.7 at 1,600 s^{-1}. In contrast to the extensive platelet spreading observed on fibronectin at all shear rates, platelet spreading on thrombospondin occurred only sporadically and at high shear rates. GPIa-IIa, GPIIb-IIIa, GPIV, and the vitronectin receptor, which are all proposed platelet receptors for thrombospondin, were not solely responsible for platelet adhesion to thrombospondin. These results suggest that thrombospondin may play a dual role in adhesive processes in vivo: (*a*) it may function in conjunction with other adhesive proteins to maintain optimal platelet adhesion at various shear rates; and (*b*) it may serve as a modulator of cellular adhesive functions under specific microenvironmental conditions. (*J. Clin. Invest.* 1993. 92:288–296.) Key words: thrombospondin • platelet adhesion • flow • integrins

Introduction

The interaction of platelets with adhesive macromolecules in the vascular subendothelium is a key initiating event in the maintenance of hemostasis. Several specific extracellular matrix (ECM) components, including collagen (COL),[1] fibronectin (FN), and vWf (e.g., references 1 and 2), and their respective cell surface glycoprotein (GP) receptors (e.g., references 3 and 4) have been implicated in mediating platelet adhesion. Of particular significance in evaluating platelet adhesive reactions has been the development of perfusion systems that allow exposure of a selected substratum to blood under well defined flow conditions (5–7). Indeed, certain platelet adhesive reactions of known physiologic importance have only been demonstrable under flow conditions (8).

Thrombospondin (TSP), a 450-kD multifunctional glycoprotein, has many features in common with ECM constituents known to mediate platelet adhesion. It is secreted from platelet α-granules (9) and can associate with the cell surface (10) in a manner indicative of the presence of specific TSP receptors. The inhibition of the secondary phase of platelet aggregation by anti-TSP provides support for a role of TSP in cell–cell interactions (11, 12). TSP is also synthesized, secreted, and incorporated into the ECM by a variety of cell types, including endothelial cells (13) and smooth muscle cells (14). In addition, TSP forms molecular complexes with many platelet adhesive proteins, including fibrinogen (FG [15]), FN (16), COL, laminin (LAM), and vWf (17, 18). These observations suggest that TSP also may play a role in platelet-substratum adhesion. However, the evidence to support this function of TSP has been highly variable. While Tuszynski et al. (19) have demonstrated platelet adhesion to TSP, the antiadhesive properties of this molecule have been emphasized by Lahav (20, 21). Such adhesive and antiadhesive properties of TSP have been noted with other cells as well (22). The variable results obtained by investigators measuring platelet adhesion to TSP (19–21) prompted us to examine this phenomenon under both static and well defined flow conditions.

Methods

Proteins, peptides, and ECM. TSP was purified by a modification (23) of a previously published procedure (24), except 2 mM $CaCl_2$ was included in all buffers. When analyzed on 7.5% polyacrylamide gels, TSP preparations revealed one major Coomassie-stained band with a M_r of 180,000 on reduced gels. On nonreduced gels, TSP also migrated as a single major band just below the stacking gel. ELISAs showed no contamination of the TSP preparations (coated onto microtiter plates at 100 μg/ml) by FG, FN, vWf, or vitronectin, which were detectable with the antibodies used at coating concentrations of 0.1 μg/ml.

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1. *Abbreviations used in this paper:* COL III, collagen type III; F-COL, fibrillar collagen; FN, fibronectin; LAM, laminin; LMWH, low molecular weight heparin; TSP, thrombospondin.

Human FG was purified from fresh plasma by differential ethanol fractionation and depleted of FN on gelatin-Sepharose (Pharmacia LKB, Uppsala, Sweden). Human plasma FN was isolated by affinity chromatography on gelatin-Sepharose. vWf was purified from the cryoprecipitates of fresh-frozen plasma by gel filtration on Sepharose 2B (Pharmacia LKB).

GPIa-IIa and GPIIb-IIIa were purified by affinity chromatography (25, 26). Briefly, 10 U of platelets was washed with TBS containing 1 mM EDTA and 0.2% (wt/vol) glucose. The platelets were lysed at 4°C in lysis buffer (TBS containing 100 mM octyl-β-D-glucopyranoside [Calbiochem-Novabiochem Corp., La Jolla, CA], 1 mM $MgCl_2$, 1 mM $MnCl_2$, 10 μg/ml leupeptin, and 1 mM PMSF). After centrifugation at 35,000 *g* for 20 min, the lysate was precleared on a Sepharose 4B column and then applied to a KYGRGDS-Sepharose column (bed volume, 10 ml). The column was washed with the lysis buffer and the bound GPIIb-IIIa was eluted with 20 mM EDTA. The unbound material was applied to a type I collagen-Sepharose column (bed volume, 3 ml). The column was washed with lysis buffer, and the bound GPIa-IIa was eluted with 20 mM EDTA. The receptors exhibited typical properties (25, 27) including appropriate gel patterns and molecular weights.

Monoclonal antibodies were from the following sources: 6F1 (anti-GPIa-IIa, $\alpha_2\beta_1$) was from Dr. Barry Coller, State University of New York, Stony Brook, NY; LM609 (antivitronectin receptor, $\alpha_v\beta_3$) was from Dr. David Cheresh, The Scripps Research Institute, La Jolla, CA: 2G12 and HP1 (anti-GPIIb-IIIa, $\alpha_{IIb}\beta_3$) were from Dr. Virgil Woods, University of California at San Diego, La Jolla, CA, and Dr. W. Nichols, Rochester, MN, respectively; C17 (anti-GPIIIa, β_3) was from Albert von dem Borne, Amsterdam, The Netherlands; 176 D7 (anti-GPIa, α_2) was from Dr. H. R. Gralnick, Bethesda, MD, anti-GPIIa (β_1) was from Dr. Carolyn Damski, San Francisco, CA; and AK2 (anti-GPIb) was from Dr. M. Berndt, Sydney, Australia. OKM5 was obtained from Ortho Diagnostics Systems, Inc. (Raritan, NJ). Monoclonal antibodies to TSP were derived from the same hybridoma fusion that yielded TSPI-1 (28).

For ECM, human umbilical vein endothelial cells were isolated with 0.05% trypsin and 0.02% EDTA (Gibco, Parsley, UK) as previously described (29) and cultured in 80% RPMI 1640 (Gibco, Parsley, UK) and 20% pooled human serum. After reaching confluence, the cells were subcultured on glass coverslips precoated with gelatin (E. Merck AG, Darmstadt, Germany), for 5–7 d. The confluent cell monolayer was then exposed to 0.1 M NH_4OH for 30 min at 22°C, with gentle shaking to remove cells, and the resulting matrix was washed extensively with TBS containing 2 mM $CaCl_2$ (TBS-Ca). The matrix was then incubated with TSP in TBS-Ca at 22°C for 2 h, and blocked with 1% human albumin (Sigma Immunochemicals, St. Louis, MO) before use.

Platelet rich plasma (PRP), washed platelets, and reconstituted blood. Fresh blood from normal donors was anticoagulated with a 1:10 vol of 110 mM trisodium citrate or 20 U/ml low molecular weight heparin (LMWH; Kabi Vitrum, Lund, Sweden). The blood was centrifuged (120 *g*, 20 min, 22°C) to pellet red blood cells, and the resulting PRP (CIT-PRP or HEP-PRP) was recentrifuged to remove residual red blood cells. Platelets were pelleted from PRP supplemented with 20 μg/ml PGE_1 by centrifugation (1,200 *g*, 20 min, 22°C), resuspended in buffer A (modified Krebs Ringer buffer [10 mM Hepes, 20 mM $NaHCO_3$, 120 mM NaCl, 4 mM KCl, 2 mM Na_2SO_4, and 0.1% glucose, pH 7.4]) containing 0.1% BSA (Sigma Immunochemicals) and 20 μg/ml PGE_1, and washed on a 40-ml column of Sepharose CL-2B (Pharmacia, LKB) preequilibrated with chelex-treated buffer A containing 0.1% BSA and 20 ng/ml PGE_1. In some experiments, platelets were resuspended in plasma and radiolabeled with 1 mCi $Na^{51}CrO_4$ at 37°C for 1 h before washing by gel filtration.

Reconstituted blood was prepared as previously described (30), with slight modifications. Briefly, HEP-PRP was diluted 1:1 with 20 mM $NaHCO_3$ buffer, pH 6.0, containing 128 mM NaCl, 4 mM KCl, 2 mM Na_2SO_4, 0.1% glucose, and 0.1% human albumin. Platelets were pelleted (1,200 *g*, 20 min, 22°C), washed twice in the same buffer, and resuspended in buffer A containing 4% (wt/vol) human albumin to a platelet concentration of 3.3×10^8/ml. Red cells were washed three times with isotonic saline containing 0.1% glucose (3,000 *g*, 10 min, 22°C) and centrifuged (4,500 *g*, 12 min, 22°C) to obtain packed cells. Blood was then reconstituted for each perfusion by mixing 6 ml of packed red cells with 9 ml of platelet suspension (hematocrit, 0.4; final platelet concentration, 2×10^8/ml).

Substrata coating of petri dishes and coverslips. Petri dishes (35 mm, No. 1008; Falcon Labware, Oxnard, CA) were coated with 0.7 ml protein in TBS-Ca, or PBS at 22°C for 2 h and then washed and post-coated with 0.3% of heat-inactivated BSA for 1 h. 96-well flat-bottomed plates (Immulon; Dynatech Labs Inc., Chantilly, VA) were coated with 0.1 ml of protein and blocked with BSA as described above. Square glass coverslips (18 mm) were cleaned by soaking overnight in 80% ethanol, rinsed in distilled deionized water, and dried thoroughly. An airbrush (model 100; Badger Meter, Inc., Milwaukee, WI) was then used to spray a fine mist of TSP, FG, FN, or vWf in volatile ammonium acetate, or fibrillar equine collagen (F-COL, collagen reagent Horm; Hormon-Chemie, Munich, Germany) to obtain a protein density of 15 μg/coverslip (5). COL type III (Sigma Immunochemicals) was dissolved in acetic acid before spraying. Alternatively, glass coverslips were coated with TSP in TBS-Ca or with mouse LAM (Sigma Immunochemicals) in PBS by adsorption at 22°C for 2 h. When generating composite substrate, the protein-coated coverslips were incubated with TSP in TBS-Ca for 2 h at 22°C. All coverslips were postcoated with 1% human albumin in TBS-Ca before use.

Static adhesion assays. 2.5 ml PRP or washed platelets (2×10^8/ml) was added to protein-coated coverslips or coated petri dishes and incubated at 37°C for 30 min. The nonattached platelets were removed by aspiration and the coverslips or petri dishes washed three times with 10 mM Hepes, 150 mM NaCl, pH 7.4 (HBSS), containing the selected divalent cations (wash buffer). The adherent platelets were fixed overnight in 0.5% glutaraldehyde in PBS, dehydrated with methanol, and stained with May-Grunwald/Giemsa (5). Cell adhesion was evaluated using a light microscope with a micrometer.

Perfusion adhesion assays. Platelet adhesion under nonpulsatile flow conditions was performed using a parallel plate perfusion chamber as previously described (5). Duplicate protein-coated glass coverslips were inserted in the chamber and rinsed with 15 ml of wash buffer. 15 ml whole blood was prewarmed at 37°C for 5 min and then recirculated through the chamber for 5 min at wall shear rates ranging from 50 to 2,250 s^{-1}. The coverslips were removed from the chamber, rinsed with wash buffer, then fixed and stained with May-Grunwald/Giemsa. The extent of coverage by adherent platelets was evaluated by light microscopy of stained glass coverslips at a magnification of 1,000, aided by an image analyzer (AMS 40-10; Analytical Measuring Systems, Saffron Walden, UK), which was interfaced to the microscope. Platelet adhesion, expressed as percent surface covered with platelets, is the average of 30 fields/coverslip.

Ligand binding to receptor-containing liposomes. Phosphatidylcholine liposomes were prepared and assayed for their attachment to TSP as described (31). Liposomes containing the receptors were prepared by mixing the isolated GPIIb-IIIa or GPIa-IIa with egg yolk phosphatidylcholine (Sigma Immunochemicals) and [^{3}H]phosphatidylcholine (DuPont-NEN Boston, MA), followed by dialysis (24 h at 4°C) against TBS containing 1-mM concentrations of the selected divalent ions. The liposomes were diluted with TBS containing 2.5 mg/ml BSA and the appropriate divalent ion and added to protein-coated 96-well flat-bottomed plates. After an overnight incubation at 4°C, the plates were washed with TBS to remove unbound ligand. Bound radioactivity was solubilized in 1% SDS/0.1 N NaOH and counted in a beta counter.

Results

The nonadhesive and antiadhesive properties of TSP. In initial experiments, the capacity of TSP to support the adhesion of

platelets in citrated whole blood or PRP was compared with that of several proteins with known adhesive properties. Platelet adhesion was measured at $0 s^{-1}$, and at shear rates determined to be optimal for each adhesive protein. Since earlier studies had expressed platelet adhesion as either number of adherent platelets/unit area under static conditions or percent surface coverage under flow conditions, we used these two conventions for data reduction to facilitate comparison with the existing data in the literature. In flow experiments, the percent surface coverage represents contact, spread, or aggregated platelets, while in static experiments only the number of adherent platelets/unit area was recorded. When coverslips were coated with TSP, minimal platelet adhesion was observed under static conditions ($0 s^{-1}$), or under flow conditions at wall shear rates of 300 and 1,600 s^{-1} (see Table I). Whether expressed as the number of adherent platelets or the surface area covered, platelet adhesion to TSP was negligible and no platelet spreading on TSP was observed. In the same experiments, other adhesive proteins supported extensive platelet deposition; surface coverage ranged from 11% for LAM to 50–70% for ECM. The presence of TSP on the coverslips was documented by coating the coverslips with ^{125}I-TSP. Under the coating conditions used, TSP was present on the coverslips at a density of 15 μg/coverslip, which is similar to the quantities of other deposited adhesive proteins; and, after exposure of the coverslips to platelets in CIT-PRP for 30 min at 37°C, > 75% of the ^{125}I-TSP remained on the coverslips. Thus, under the conditions of these initial analyses, TSP failed to support platelet adhesion.

These results prompted an investigation of the capacity of TSP to modulate platelet–substratum interactions. The LAM, FN, vWf, and FG substrata, and also ECM, supported platelet attachment and spreading, whereas COL III and F-COL also supported the formation of platelet aggregates. Pretreatment of the nonthrombogenic surfaces (FG, FN, and vWf) with TSP (0.5 mg/ml) resulted in significant inhibition of platelet adhesion (Table II). This inhibition was concentration dependent, occurred under both static and flow conditions, and ranged from 37 to 70%. On the thrombogenic surfaces, the inhibition of platelet adhesion by TSP was either donor dependent (COL III) or modest (F-COL). Platelet adhesion to LAM and ECM was only inhibited by TSP under static conditions. To exclude the possibility that the inhibition of platelet adhesion by TSP was due to displacement of primary proteins on the coverslips by the TSP preparation, ^{125}I-labeled primary proteins were sprayed onto coverslips. After subsequent incubation with 0.5 mg/ml TSP or buffer, > 90% of the radioactivity (calculated relative to the buffer control) remained associated with the TSP-treated coverslips. More ^{125}I-TSP (1.5–3.5-fold) associated with the coverslips coated with platelet adhesive proteins than with albumin, but the amount of TSP bound did not determine its potency as an inhibitor of platelet adhesion (e.g., more TSP associated with F-COL than FG, but platelet adhesion to F-COL was minimally affected by TSP).

Adhesive properties of TSP. Platelet adhesion was measured in blood anticoagulated with LMWH to maintain higher divalent cation concentrations. In contrast to the results obtained above in citrated blood, TSP now supported platelet adhesion (Fig. 1). At 0 and 300 s^{-1}, platelet adhesion to TSP was less extensive than to FN. When the shear rate was increased from 300 to 1,600 s^{-1}, platelet adhesion to FN decreased by 76% while adhesion to TSP increased significantly and exceeded the maximal level obtained with FN. Further increase in shear rate to 2,250 s^{-1} caused a decline in platelet

Table I. Adhesion of Platelets in Citrated Blood to Purified Proteins and Endothelial Cell Matrix

Protein	Platelet adhesion		
	0 s^{-1} platelets	300 s^{-1} coverage	1,600 s^{-1} coverage
	per mm²	%	%
Albumin	150	0.1	0.2
TSP	240	0.6	0.8
FG	1,260	37.1	—
FN	1,090	24.8	—
Laminin	690	11.4	—
vWF	450	—	45.3
Collagen III	790	—	26.0
Fibrillar collagen	850	—	27.5
ECM	1,620	49.7	70.0

ECM or coverslips sprayed with adhesive proteins (1 mg/ml for collagen and fibrillar collagen and 0.2 mg/ml for the others) were blocked with 1% human albumin for 1 h at 22°C. The coverslips were then incubated (30 min, 37°C) with CIT-PRP for static adhesion assay ($0 s^{-1}$), or perfused with citrated whole blood (5 min, 37°C) at wall shear rates of 300 and 1,600 s^{-1}. Platelet adhesion is expressed as number of adherent platelets/mm^2 in static adhesion assays, and percent coverage in perfusion adhesion assays.

Table II. Modulation of the Adhesion of Platelets in Citrated Blood by TSP

Primary protein	TSP	Platelet adhesion (Control)		
		0 s^{-1}	300 s^{-1}	1,600 s^{-1}
	mg/ml		%	
FG	0.1	73.0±9.5	67.3±2.1	—
	0.5	54.7±8.5	47.3±20.3	—
FN	0.1	59.3±6.5	80.0±8.7	—
	0.5	30.0±4.0	52.3±14.6	—
Laminin	0.1	69.7±17.6	108.0±7.2	—
	0.5	62.0±7.0	105.7±13.2	—
vWF	0.1	91.3±11.8	—	90.7±12.5
	0.5	55.0±18.0	—	62.7±16.2
Collagen III	0.1	92.6±8.7	—	72.0±42.4
	0.5	63.7±24.8	—	46.3±38.2
Fibrillar collagen	0.1	96.0±12.0	—	—
	0.5	89.6±2.5	—	83.3±4.5
ECM	0.1	74.3±22.5	104.3±4.5	99.7±6.0
	0.5	62.0±21.5	99.7±3.5	102.3±2.5

Isolated ECM or glass coverslips, sprayed with primary proteins (0.2 mg/ml for FG and FN; 0.1 mg/ml for laminin and vWF; and 1 mg/ml for collagen III and fibrillar collagen), were incubated with TSP in TBS-Ca^{2+} for 2 h at 22°C. For controls, coverslips were incubated with buffer alone. Platelet adhesion was measured as described in the legend to Table I. Adherent platelets, expressed as percent control, is the mean±SD of three separate experiments.

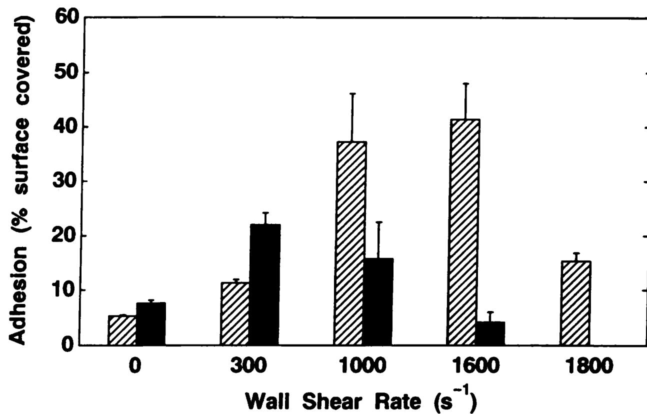

Figure 1. Comparison of platelet adhesion to TSP and FN in heparinized blood. Glass coverslips were coated by adsorbing 0.2 mg/ml of TSP (*hatched bars*) or FN (*closed bars*) for 2 h at 22°C and blocked with 1% human albumin. Platelet adhesion was measured as described in the legend to Table I, using blood anticoagulated with LMWH. Platelet adhesion, expressed as percent surface covered with platelets, is the mean±SD of quadruplicate assays. The percent coverage varied significantly among experiments, but similar patterns were obtained in all experiments.

adhesion to TSP. Representative micrographs of the platelets adherent to TSP are shown in Fig. 2. While platelet spreading on FN was > 60% of the total platelet coverage at 300 and 1,000 s^{-1} (Fig. 2 *A*), < 10% spreading occurred on TSP at 300 s^{-1} (Fig. 2 *B*). In some experiments, platelets did spread on TSP at 1,000 and 1,600 s^{-1} (Fig. 2 *C*), but this occurred infrequently and may be donor dependent. Specificity of the adhesion of platelets to TSP was indicated by the observation that two monoclonal antibodies to TSP (9D3 and 14E7) blocked adhesion by > 50%, whereas they had no effect on platelet adhesion to fibronectin.

To confirm the observed platelet adhesion to TSP was divalent ion dependent and to rule out contributions from LMWH, citrate, or plasma components to this process, experiments were performed with washed platelets under static conditions. FN and BSA were used as a positive and negative control, respectively (Fig. 3). In the absence of divalent cations, TSP did not support platelet adhesion and inhibited the nonspecific interaction of platelets with BSA (1.24×10^5 platelets/cm^2), while FN promoted platelet adhesion twofold. The addition of Ca^{2+} alone had no significant effect on platelet adhesion to FN, caused a slight increase in platelet adhesion to TSP, and decreased the nonspecific adhesion to BSA. Platelet adhesion to FN was stimulated eightfold in the presence of Mg^{2+} alone, and this effect was attenuated by the addition of Ca^{2+}. In contrast, Mg^{2+} alone had no significant effect on platelet adhesion to TSP; with both Ca^{2+} and Mg^{2+} present, platelet adhesion to TSP was 50-fold higher than to BSA (Fig. 3). This increment was not affected by 20 U/ml LMWH (2.25×10^5 platelets/cm^2 vs. 2.30×10^5 platelets/cm^2 in the presence or absence of 20 U/ml LMWH) and was inhibited by < 20% in the presence of 200 U/ml LMWH. Tuszynski and Kowalska (32) observed that $MnCl_2$ greatly enhanced platelet adhesion to TSP. In our hands, platelet adhesion to TSP occurred more consistently in the presence of 1 mM Ca^{2+} and Mg^{2+} ($8.1 \pm 4.5 \times 10^5$ platelets/cm^2) than in the presence of 100 μM Mn^{2+} ($6.2 \pm 8.7 \times 10^5$

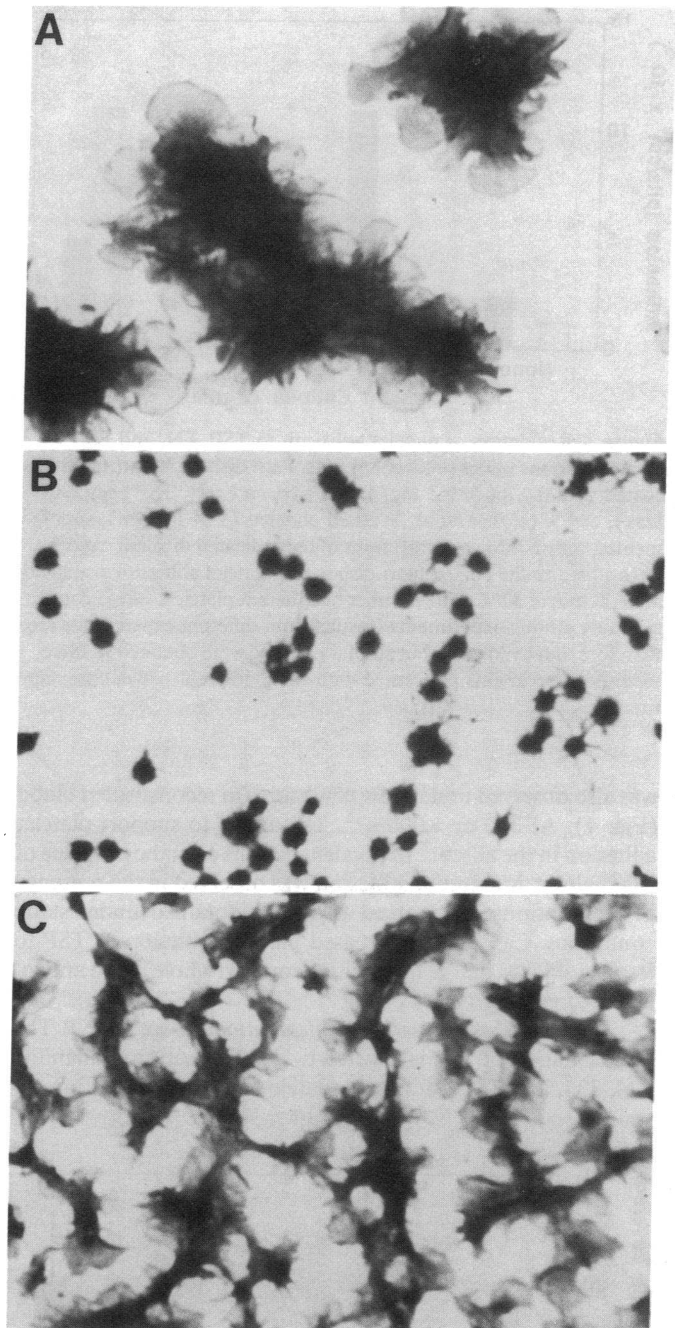

Figure 2. Light micrographs of adherent platelets on TSP and FN. Coverslips from representative experiments were photographed at 100×. (*A*) Adherent platelets on FN-coated substrata at 300 s^{-1}. (*B*) Adherent platelets on TSP-coated substrata at 300 s^{-1}. (*C*) Adherent platelets on TSP-coated substrata at 1,000 s^{-1}.

platelets/cm^2). In experiments in which Mn^{2+} promoted adhesion, the platelets adhered as large aggregates rather than as the single cells observed in the presence of Ca^{2+} and Mg^{2+} (Fig. 2 *B*). Furthermore, PGE_1/theophylline had no effect on platelet adhesion promoted by Ca^{2+} and Mg^{2+} ($7.4 \pm 4.5 \times 10^5$ platelets/cm^2) but significantly decreased platelet adhesion supported by Mn^{2+} ($2.0 \pm 3.3 \times 10^5$ platelets/cm^2).

The divalent ion dependence of platelet adhesion of TSP

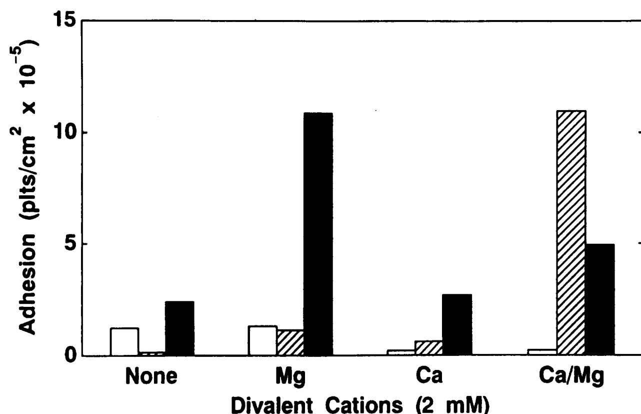

Figure 3. Regulation of platelet adhesion to TSP, FN, and BSA by divalent cations under static conditions. Petri dishes (35 mm) were coated with 0.7 ml of 0.2 mg/ml BSA (*open bars*), TSP (*hatched bars*), or FN (*closed bars*). Washed platelets (2×10^8/ml), supplemented with 2-mM concentrations of the indicated divalent cations, were added to the coated petri dishes and platelet adhesion evaluated after 30 min at 37°C. The number of adherent platelets varied considerably among experiments (results from different experiments are, therefore, not averaged), but similar patterns were obtained in three separate experiments performed with three different platelet preparations.

was also observed under flow conditions in reconstituted blood (Fig. 4). At 300 or 1,000 s^{-1}, TSP failed to support platelet adhesion in the absence of divalent cations or in the presence of Mg^{2+} alone. Maximum adhesion was observed in the presence of both cations. In contrast to results obtained under static conditions, Ca^{2+} alone increased platelet adhesion to TSP to levels similar to that of the positive control (whole blood anticoagulated with LMWH).

Platelet adhesion and the conformational state of TSP. The conformation of TSP is affected by divalent cation availability (24, 33), and TSP binding to platelets is also divalent ion dependent (10). Thus, the regulation of platelet adhesion to TSP by divalent cations could be dependent upon the conformational state of the TSP molecule and/or upon the divalent cation requirement of its cell surface receptor(s). These possibilities were considered in experiments that compared platelet adhesion in a low Ca^{2+} environment of CIT-PRP vs. the normal Ca^{2+} environment of HEP-PRP. TSP adsorbed onto coverslips in the presence of EDTA failed to support platelet adhesion in either CIT-PRP and HEP-PRP under static conditions (Fig. 5). EDTA-treated TSP also failed to support platelet adhesion (< 0.5% platelet coverage) at wall shear rates ranging from 50 to 1,600 s^{-1}. When TSP was adsorbed onto coverslips in the presence of 2 mM Ca^{2+}, platelet adhesion occurred in HEP-PRP but not in CIT-PRP. To determine if the low divalent cation concentrations of CIT-PRP induced changes in the TSP molecule, the Ca-conformer of TSP on the coverslips was preincubated with buffer containing low Ca^{2+} (30 μM) to mimic the free calcium concentration in CIT-PRP. Under these conditions, the capacity of TSP to support the adhesion of platelets in HEP-PRP diminished by 75% (Fig. 5). These results suggest that, at low divalent cation concentrations, Ca^{2+} dissociates from TSP to form a conformer that does not support platelet adhesion. The time course for this putative conformational change is rapid (Fig. 6). At 37°C, a 53% reduction in the capacity of TSP to support platelet adhesion was observed within 15 min after being placed in a low Ca^{2+} environment. In studies in which Ca^{2+} concentrations were varied (not shown), the conformational transition occurred at ~ 0.25 mM. At this concentration, platelet adhesion was reduced to 28% of the level obtained in 2 mM Ca^{2+}.

Nature of the TSP receptor. The effects of antibodies to selected platelet membrane glycoproteins on divalent ion dependent–platelet adhesion to TSP were assessed under static conditions (Table III). LM609, a monoclonal to the vitronectin receptor ($\alpha_v\beta_3$), which inhibits endothelial cell adhesion to

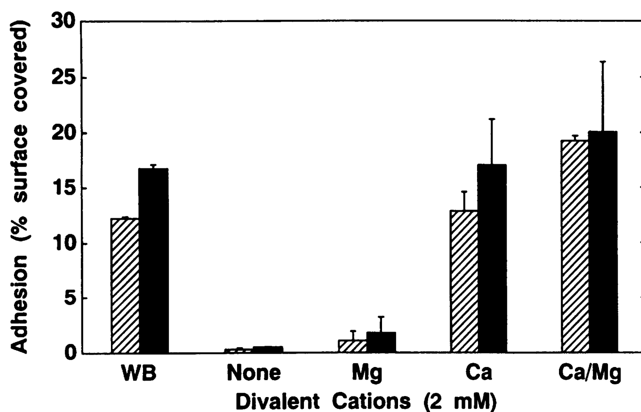

Figure 4. Regulation of platelet adhesion to TSP by divalent cations under flow conditions. Whole blood (WB), or reconstituted blood supplemented with 2-mM concentrations of the indicated divalent cations, were perfused at 300 s^{-1} (*hatched bars*) or 1,000 s^{-1} (*closed bars*). Platelet adhesion, expressed as percent surface covered with platelets, is the mean±SD of quadruplicate determinations from a representative experiment. Similar results were obtained in three separate experiments.

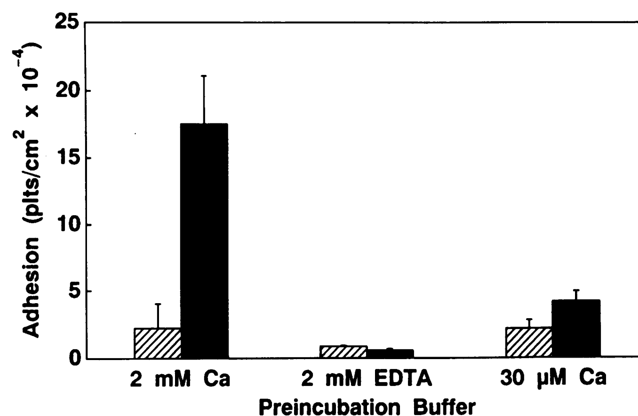

Figure 5. Effect of calcium on TSP conformation and its relationship to platelet adhesion. Glass coverslips were treated in three ways: coated with TSP in buffer containing 2 mM EDTA, and preincubated in the same buffer; coated with TSP in buffer containing 2 mM Ca^{2+}, and preincubated in the same buffer; coated with TSP in buffer containing 2 mM Ca^{2+} and preincubated in buffer containing 30 μM Ca^{2+}. All preincubations were carried out at 37°C for 1 h. The adhesion assay was performed under static conditions using CIT-PRP (*hatched bars*) or HEP-PRP (*closed bars*). Platelet adhesion is the mean±SD of triplicate determinations from one experiment. Similar results were obtained in three separate experiments.

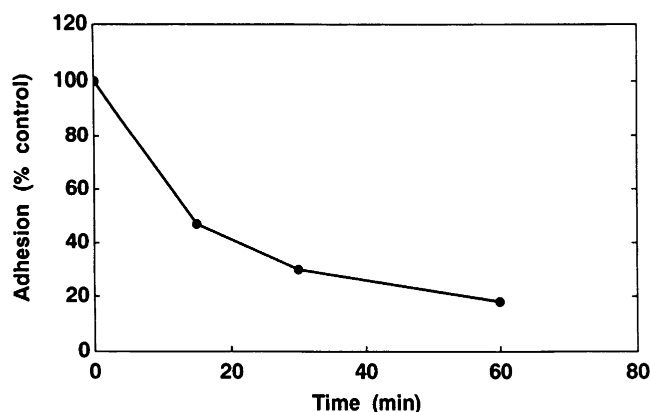

Figure 6. Time course for the conversion of TSP from the adhesive to the nonadhesive conformation. Glass coverslips were coated with TSP in buffer containing 2 mM Ca^{2+} and preincubated at 37°C in the same buffer for 60 min (control), or in buffer containing 30 μM Ca^{2+} for 15, 30, or 60 min. The adhesion assay was performed under static conditions using HEP-PRP. Platelet adhesion, expressed as percent control, is the average of two experiments.

TSP (34), had no effect on platelet adhesion. OKM5, an antibody to GPIV (CD36), enhanced platelet adhesion to TSP, presumably due to its platelet-activating activity (35). To circumvent this effect, PGE_1/theophylline was included in the assays. Under this condition, OKM5 had no effect on platelet adhesion to TSP. 6F1, a monoclonal to GPIa-IIa ($\alpha_2\beta_1$) (36), inhibited platelet adhesion to TSP by 58% at a concentration of 200 μg/ml. This same antibody completely blocked GPIa-IIa–mediated platelet adhesion to collagen, a known ligand for this integrin (27), at concentrations as low as 10 μg/ml. 2G12, a monoclonal to GPIIb-IIIa ($\alpha_{IIb}\beta_3$), inhibited the adhesion of washed platelets to TSP by 59.3%. Under the same conditions, 2G12 inhibited platelet adhesion to FG, a known GPIIb-IIIa ligand, by 77.7%. In contrast to its effects on platelet adhesion to TSP in buffer, 2G12 was not inhibitory in plasma. As shown in Fig. 7, in buffer, 2G12 inhibited the interaction of platelets with TSP, FN, and FG by 59, 47, and 74%, respectively. Although 2G12 also inhibited the adhesion of platelets to FG and FN by > 60% in plasma, it had no significant effect on platelet adhesion to TSP in the plasma environment. A second GPIIb-IIIa antibody (HP1) also had no effect in perfusion studies performed in whole blood at wall shear rates of 300–1,000 s^{-1}. In the perfusion studies, antibodies (see Methods) to GPIa, GPIIa, GPIIIa, and GPIb also had no effect on platelet adhesion to TSP, whereas, in parallel experiments, the concentrations of these antibodies used gave optimal inhibition of platelet adhesion to known ligands of their target receptors.

Table III. Effect of Antibodies on Platelet Adhesion to TSP under Static Conditions

Target protein	Antibody	Platelet adhesion (Control) TSP	FG	COL I
		%		
GPIV	OKM5 (10 μg/ml)	110.9±5.6	—	—
$\alpha_v\beta_3$	LM609 (1/50)	109.0±12.3	—	—
GPIa–IIa	6F1 (10 μg/ml)	135.0±14.1	—	0
	(50 μg/ml)	80.2±29.1	—	0
	(100 μg/ml)	64.0±3.6	—	—
	(200 μg/ml)	42.0±1.4	—	—
GPIIb–IIIa	2G12 (1/50)	40.7±11.0	22.3±3.6	—

96-well microtiter plates were coated with 50 μg/ml protein and blocked with 0.3% heat-inactivated BSA. The adhesion of chromium-labeled platelets preincubated with the indicated concentrations of purified antibodies (6F1 or OKM5) or ascites fluid (2G12 or LM609) was measured after 30 min at 37°C. Platelet adhesion, expressed as percent control, is the mean±SD of three to six experiments. With OKM5, platelet adhesion was performed in the presence of PGE_1 and theophylline to prevent platelet aggregation.

To further examine the role of GPIa-IIa and GPIIb-IIIa as TSP receptors, these membrane proteins were purified and reconstituted into liposomes. The function and specificity of GPIIb-IIIa and GPIa-IIa liposomes is evidenced by their selective interaction with FG and COL I, respectively (Fig. 8). Similar to the data obtained with platelets, the Ca-depleted conformer of TSP completely failed to support the binding of either GPIIb-IIIa or GPIa-IIa liposomes to TSP, even though the binding assays were performed in the presence of divalent ions. The failure of GPIIb-IIIa liposomes to interact with the Ca-depleted conformer of TSP confirms previous data, which demonstrated that such liposomes did not bind TSP that was immobilized in the absence of divalent cations (25). The binding of the GPIa-IIa liposomes to the Ca-conformer of TSP was not supported by Ca^{2+} and Mg^{2+}, and the addition of Mn^{2+} caused only a minimal increase. In contrast, the binding of GPIIb-IIIa liposomes to the Ca-conformer of TSP increased fivefold in the presence of Ca^{2+} and Mg^{2+}, and eightfold when Mn^{2+} was added as well. 2G12 abolished the binding of GPIIb-IIIa liposomes to FG, and inhibited their binding to TSP by 60%.

Discussion

In this study, we have sought to evaluate the platelet adhesive properties of TSP under both static and flow conditions. Our results indicate that platelet adhesion to TSP is uniquely and

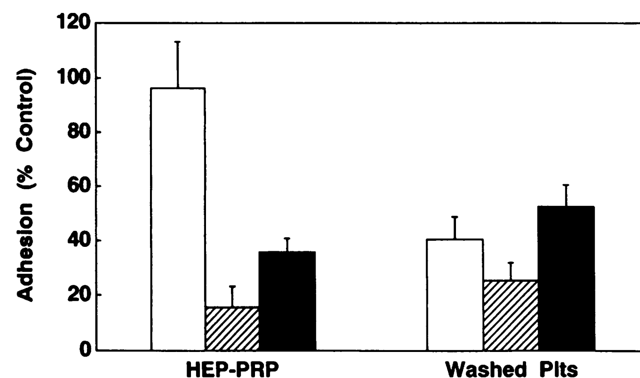

Figure 7. Inhibitory effects of a monoclonal antibody to GPIIb-IIIa on the adhesion of platelets in plasma or buffer. Washed platelets (2 × 10^8/ml) supplemented with 2 mM each of $CaCl_2$ and $MgCl_2$, or HEP-PRP, were added to 35-mm petri dishes coated with TSP (*open bars*), FG (*hatched*), or FN (*closed bars*). After 30 min at 37°C, adherent platelets were stained and evaluated by light microscopy. The monoclonal antibody was 2G12. Platelet adhesion, expressed as percent control, is the mean±SD of three different experiments.

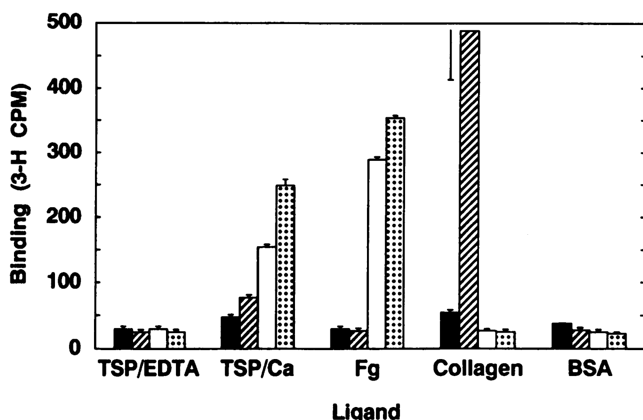

Figure 8. The interaction of purified GPIa-IIa and GPIIb-IIIa with adhesive proteins. 96-well plates were coated with 20 μg/ml TSP in TBS containing 2 mM $CaCl_2$ or 5 mM EDTA, or with 20 μg/ml FG or COL I in PBS. After blocking with 0.3% heat-inactivated BSA in the appropriate buffer, the binding of ^{3}H-labeled liposomes containing GP Ia-IIa (*hatched* and *closed bars*) or GP IIb-IIIa (*open* and *dotted bars*), supplemented with 1 mM each of $CaCl_2$ and $MgCl_2$ (*open* and *closed bars*) or $CaCl_2$, $MgCl_2$, and $MnCl_2$ (*hatched* and *dotted bars*), was measured as described in the Methods section. Results are mean±SD of triplicate determinations.

exquisitely sensitive to divalent cations. While TSP exhibited nonadhesive or even antiadhesive properties (blocking platelet adhesion to known adhesive substrata) under low divalent ion conditions, it effectively supported platelet adhesion at the normal divalent ion conditions available in heparinized blood. The divalent cation dependence of platelet adhesion to TSP appears to clarify disparities in previously reported data. Lahav (21) demonstrated the antiadhesive properties of TSP in a medium with divalent cation concentrations similar to that found in citrated blood, while the adhesion-promoting activity of TSP described by Tuszynski et al. (19) was in a medium containing high divalent cation concentrations. The conformational state of TSP, which is regulated by divalent cations (see below), also may have contributed to the nonadhesive properties of this molecule observed with other cell types, including endothelial cells (20, 34, 37, 38). Superimposed upon the stringent divalent ion requirements, shear rate played an important role in platelet adhesion to TSP. While vWf functions predominantly at high shear rates in vivo (8), TSP supported adhesion at intermediate shear rates of 300–1,600 s^{-1}. The different optimal shear rates observed for platelet adhesion to TSP and FN suggests that a combination of these proteins may be necessary to support maximal platelet adhesion at these intermediate shear rates.

The specificity of the adhesion of platelets to TSP at the divalent ion conditions in blood is supported by the following observations. (*a*) The divalent cation requirements for platelet adhesion to TSP are unique since all the other proteins tested effectively supported platelet adhesion in citrated blood. (*b*) The morphology of platelets on TSP differs from that on FN and other adhesive proteins in that, at low shear rates, no platelet spreading occurred on TSP. (*c*) TSP monoclonal antibodies significantly inhibited platelet adhesion to TSP. Taken together, these observations would appear to rule out the possibility that the adhesive properties of TSP described in this study are due to contamination.

The stringent requirement of TSP–platelet interactions for Ca^{2+} arises, at least in part, from the effect of this cation on the conformation of TSP (24, 33, 39). The type 3 repeats adjacent to the carboxy-terminal globular region of TSP contain at least 12 Ca^{2+}-binding sites, with dissociation constants ranging from 50 to 120 μM (38, 40, 41). By electron spin microscopy (24), electron spin resonance (33), and dynamic light scattering (39), removal of Ca^{2+} induces a conformational change in the carboxy-terminal domain of TSP. The type 3 repeats of TSP also contain free thiols and labile disulfide bonds that are partially protected by Ca^{2+}, but EDTA chelation of Ca^{2+} enhances intramolecular thiol-disulfide isomerization (42). Recently, Sun et al. (38) demonstrated that bovine aortic endothelial cell attachment to intact TSP occurred via a divalent cation-dependent and Arg-Gly-Asp (RGD)-dependent mechanism. Disulfide bond reduction in TSP enhanced its capacity to support RGD-dependent cell attachment and spreading, but abolished the Ca^{2+} requirements of TSP for cell adhesion. This phenomena was attributed to a disulfide-regulated exposure of a cryptic RGD sequence in the type 3 repeat of TSP. Furthermore, the observation that protein disulfide isomerase activity is secreted by activated platelets (43) indicates that this enzyme may regulate TSP adhesive properties at the site of injury by exposing RGD sequences. However, in our hands, platelet adhesion to intact TSP was not inhibitable by Gly-Arg-Gly-Asp-Ser-Pro (data not shown), but was highly regulated by Ca^{2+}.

While it is our experience that TSP in solution can be readily transformed from the Ca-deplete to the Ca-replete conformer by addition of Ca^{2+} (10), this conversion did not occur with immobilized TSP. Moreover, the transition of immobilized TSP to the Ca^{2+}-depleted conformer was rapid, with a half-time of ~ 15 min at 37°C. The capacity of TSP to change from adhesive to nonadhesive states allows the molecule to accommodate both cell adhesion and migration. Although the precise Ca^{2+} concentration at which the transition occurs was not determined, events such as Ca^{2+} chelation by highly negatively charged proteoglycans, or Ca^{2+} displacement by the interaction of other matrix components with regions close to the Ca^{2+}-binding domain of TSP, could lower local Ca^{2+} concentrations to a level that favors these conformational changes. Moreover, the conformational changes may be affected not only by changes in local divalent cation concentrations but also by other mechanisms, such as by the interaction of matrix components with non-Ca^{2+}-binding domains of TSP. These speculations are supported by a two-step model proposed by Leung et al. (44) for the interaction of TSP with GPIV, in which low affinity TSP–GPIV interactions result in conformational changes in TSP that lead to the exposure of high affinity binding sites for GPIV. However, the capacity of a small GPIV peptide (which presumably interacts with low affinity sites on TSP) to augment rather than inhibit TSP binding to GPIV suggests that the low affinity binding domain on TSP is not readily accessible to membrane associated GPIV.

Both Ca^{2+} and Mg^{2+} are required for significant TSP–platelet interactions. The only difference in the divalent ion requirements for platelet adhesion to TSP in blood vs. a washed cell system is related to the effect of Mg^{2+}. The capacity of Ca^{2+} alone to augment platelet adhesion in reconstituted blood (Fig. 4), but not with washed platelets (Fig. 3), may simply be due to the fact that red blood cells in reconstituted blood provide sufficient Mg^{2+} to fulfill the Mg^{2+} requirement for optimal platelet

adhesion (since red blood cells significantly influence platelet adhesion under flow conditions [5], experiments to verify this interpretation could not be performed). Thus, our results are consistent with previous reports, which showed that the interaction of TSP with human endothelial and smooth muscle cells only occurred when TSP was adsorbed in the presence of Ca^{2+} and the cell suspension contained both Ca^{2+} and Mg^{2+} (34). The requirement for both Ca^{2+} and Mg^{2+} is also consistent with the optimal conditions for interaction of TSP with the platelet surface (10, 28).

As noted by Murphy-Ullrich (45), the identity of the platelet receptor(s) for TSP remains unresolved. While our data provide some insights into this issue, they also emphasize the complexity of the TSP receptors on platelets. GPIV has been proposed as the TSP receptor on platelets, partly based upon the inhibitory effects of OKM5 on TSP–platelet and TSP–purified GPIV interactions (44, 46, 47). We have been unable to demonstrate an effect of this antibody on TSP–platelet interactions (35); and, in the present study, we found no effect of OKM5 on platelet adhesion to TSP. Moreover, normal TSP surface expression on GPIV-deficient platelets has been reported (48, 49). The identification of $\alpha_v\beta_3$ as a TSP receptor is based on the interaction of the receptor from endothelial cell (34) and platelet (50) lysates with immobilized TSP, and the inhibition of endothelial cell adhesion to TSP (34) and reduced TSP (38) by anti-$\alpha_v\beta_3$ (LM609). This antibody had no effect on platelet adhesion to TSP (even though the activity of the antibody preparation was independently verified). GPIa-IIa has also been implicated in TSP–platelet interactions as GPIa-IIa–deficient platelets also were deficient in TSP (51) (analogous to the fibrinogen deficiency within GPIIb-IIIa–deficient platelets). Moreover, Tuszynski and Kowalska (32) found that 6F1, a monoclonal to GPIa-IIa, inhibited platelet adhesion to TSP. This observation was reproduced in our study. However, very high concentrations of 6F1 were required to inhibit this interaction, much higher than those needed to completely abrogate collagen-platelet adhesion. Furthermore, GPIa-IIa liposomes failed to interact with TSP, indicating that, if TSP does interact with GPIa-IIa, it must do so by a mechanism distinct from collagen binding to this receptor. Finally, in a preliminary analysis, platelets from a patient lacking GPIa exhibited normal adherence of TSP (11.6% surface coverage for the GPIa-deficient platelets vs. 11.4% for control platelets at 300 s^{-1}). Thus, it is unlikely that GPIa-IIa functions as an adhesion receptor for TSP.

Our results do suggest a role of GPIIb-IIIa in TSP–platelet interactions under certain conditions, as suggested by Tuszynski and co-workers (32, 52). GPIIb-IIIa antibodies partially blocked the adhesion of washed platelets to TSP, and GPIIb-IIIa liposomes interacted specifically with the Ca^{2+}-repleted but not the Ca^{2+}-depleted TSP. However, GPIIb-IIIa was not implicated in TSP–platelet interactions in the presence of plasma. With the capacity of TSP to interact readily with many plasma and matrix constituents, and of adhering platelets to secrete adhesive proteins, including TSP, which can regulate the function of matrix TSP, the nature of TSP-mediated adhesion may change continuously and multiple receptors are likely to be involved. Such redundancy would obscure the involvement of GPIIb-IIIa in plasma and permit the adhesion of GPIIb-IIIa–deficient platelets to TSP. Consistent with this conclusion is our preliminary analysis of the adhesion of thombasthenic platelets to TSP. At 800 s^{-1} in whole blood, the thrombasthenic platelets showed an adherence (24.8% surface coverage) similar to that of normal platelets (29.4% surface coverage) when analyzed in parallel. Overall, these results suggest that none of the receptors studied, including GPIIb-IIIa, is solely responsible for mediating the initial interaction of platelets with TSP, but the involvement of these receptors in subsequent stages of this process cannot be ruled out. Further studies are required to unequivocally identify the major platelet adhesion receptor(s) for TSP.

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