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Research Article

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Thrombospondin-induced Adhesion of Human Platelets

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Abstract

Washed human unactivated platelets attached and spread on thrombospondin (TSP)-coated microtiter plates. Platelet adhesion was promoted by divalent cations Mn^{2+}, Mg^{2+}, and Ca^{2+} as compared to buffer having all divalent cations complexed with EDTA. TSP-dependent adhesion was inhibited by anti–TSP fab fragments, an anti–TSP monoclonal antibody, an RGD-containing peptide, complex-specific anti–glycoprotein (GP)IIb-IIIa monoclonal antibodies (A_2A_9 or AP-2) and anti–VLA-2 monoclonal antibodies (6F1 and Gi9), but not by rabbit preimmune fab fragments, mouse IgG, an anti–GPIIIa monoclonal antibody, or monoclonal antibodies against either the human vitronectin receptor, glycocalicin, or GPIV. At saturating concentrations, anti–GPIIb-IIIa inhibited adhesion by 40–60%. Glanzman's thrombasthenic platelets, which lack GPIIb-IIIa, adhered to TSP to the same extent as anti–GPIIb-IIIa-treated normal platelets or 40–60% as well as untreated normal platelets. Antibody 6F1 (5–10 μg/ml) inhibited platelet adhesion of both normal and thrombasthenic platelets by 84–100%. Both VLA-2 antibodies also inhibited collagen-induced platelet adhesion, but had no effect on fibronectin-induced adhesion of normal platelets. These data indicate that platelets specifically adhere to TSP and that this adhesion is mediated through GPIIb-IIIa and/or VLA-2. (*J. Clin. Invest.* 1991. 87:1387–1394.) Key words: adhesion • glycoproteins • platelets • receptor • thrombospondin

Introduction

Platelet adhesion is a critical step in the process of hemostasis. When a blood vessel is ruptured, a complex cascade of events is rapidly triggered in order to seal the damaged vessel and prevent further bleeding. Initially, platelets adhere to components of the injured vessel, become activated, secrete the contents of their granules, and quickly transform the area of the injury into a highly thrombogenic surface. Subsequently, the adherent layer of platelets activates and binds more platelets, forming platelet aggregates. The platelet aggregates are procoagulant, generating locally high concentrations of thrombin, and converting platelet-bound fibrinogen into fibrin. The growing platelet fibrin plug entraps other cells, such as erythrocytes, and the resulting thrombus rapidly seals the bleeding vessel.

The elucidation of the mechanisms leading to platelet–platelet and platelet–vessel wall interactions occurring during normal hemostasis and as a consequence of pathological conditions such as atherosclerosis are of great interest. Platelet–collagen interactions play an important role in hemostasis since patients with impaired collagen-induced platelet aggregation and adhesion experience mild to severe bleeding (1). Recently, Kehrel and co-workers (2) described a patient with defective collagen-induced platelet aggregation who suffered from a life-long bleeding tendency. Both the bleeding tendency and collagen-induced platelet aggregation was corrected when, for some unknown reason, the patient's platelet thrombospondin (TSP),[1] a major platelet-secreted protein (3), was no longer degraded and glycoprotein (GP)Ia-IIa, also referred to as VLA-2, reappeared on the platelet membrane. These observations, as well as our previous work showing that TSP promotes platelet aggregation (4), suggests that TSP plays an important role in mechanisms of hemostasis involving platelet cohesion and adhesion. In this report, we further investigated the role of thrombospondin in hemostasis by showing that TSP can directly promote platelet adhesion. This adhesion is divalent cation dependent and partly mediated by the platelet–GPIIb-IIIa complex and the platelet–VLA-2 complex.

Methods

Materials. All reagents, unless specified otherwise, were purchased from Sigma Chemical Co., St. Louis, MO. The peptides, GRGDS and GRGESP, were purchased from Peninsula Laboratories, Inc., Belmont, CA. Silicone oils were obtained from William F. Nye, Inc., New Bedford, MA. Alkaline phosphatase–coupled avidin D was purchased from Vector Laboratories, Inc., Burlingame, CA. The bicinchoninic acid (BCA) protein reagent was purchased from the Pierce Chemical Co., Rockford, IL. Tritium-labeled serotonin and ^{125}I-labeled iodine were purchased from Amersham Corp., Arlington Heights, IL. 96-well microtiter plates were purchased from Costar Data Packaging, Inc., Cambridge, MA.

TSP purification. TSP was purified from Ca^{+2} ionophore A23187–activated human platelets as previously described (5).

Antibodies. The monoclonal antibodies, A_2A_9 and SSA_6, against human platelet GPIIb-IIIa were kindly provided by Dr. Joel Bennett, University of Pennsylvania School of Medicine, Philadelphia, PA. Monoclonal antibody A_2A_9 is directed against epitopes on both GPIIb and GPIIIa (6), whereas SSA_6 is specific for GPIIIa (7). Monoclonal antibody AP-2 was kindly provided by Dr. Thomas J. Kunicki, Blood Center of Southeastern Wisconsin, Milwaukee, WI. AP-2 is directed against epitopes on both GPIIb and GPIIIa (8). Monoclonal antibody LM609 was kindly provided by Dr. David Cheresh, Research Institute of Scripps Clinic, La Jolla, CA. Monoclonal antibody LM609 is directed against an RGD-specific domain of the human vitronectin receptor (9). Monoclonal antibodies 6D1, specific for platelet GPIb (10), and 6F1, specific for the human platelet–VLA-2 complex (11) were kindly provided by Dr. Barry S. Coller, Department of Medicine, State University of New York, Stony Brook, NY. Monoclonal antibody Gi9, specific against human platelet VLA-2, and monoclonal antibody

OKM5, specific against platelet GPIV, were purchased from Amac, Inc., Westbrook, ME and Ortho Diagnostics Systems, Inc., Raritan, NJ, respectively. Polyclonal rabbit anti–human TSP antibody was raised against the 180,000-mol wt chain of purified TSP eluted from an SDS gel as previously described (5). The antibody specifically recognized TSP as demonstrated by direct ELISA but did not cross-react with 200 ng of the following proteins immobilized on a microtiter dish: albumin, fibrinogen, fibronectin, collagen, and vitronectin. For example, there was no statistical difference in the reactivity of polyclonal anti–TSP IgG toward either albumin or vitronectin giving an absorbance value of 0.086±0.019 and 0.122±0.016, respectively. Fab fragments from anti–TSP and preimmune serum were prepared according to the instructions provided with a kit purchased from Pierce Chemical Co. Anti–human TSP (MAb 054) and anti–human collagen type IV (MAb1430) monoclonal antibodies were purchased from Chemicon, Temecula, CA. The commercially available MAb 054 is clone P10 developed and characterized by Clezardin et al. (12). Human vitronectin, anti–vitronectin, fibronectin, and anti–fibronectin were obtained from Calbiochem-Behring Corp., La Jolla, CA. Biotin conjugated goat anti–rabbit Fab-specific IgG was obtained from Accurate Chemical and Scientific Corp., Westbury, NY.

Protein assay. Protein concentrations were determined by the BCA protein assay, adapted for microtiter plates, as described by the Pierce Chemical Co. Bovine serum albumin (BSA) was used as standard.

Platelet isolation. Blood (100 ml) was obtained by venipuncture from normal volunteers and anticoagulated with acid citrate dextrose (National Institutes of Health formula). Prostaglandin E_1 (PGE$_1$) was added to a final concentration of 1 μM. Platelet-rich-plasma (PRP) was prepared by centrifuging the blood at room temperature for 20 min at 150 g. Platelets were pelleted by centrifuging the PRP at room temperature for 20 min at 3,000 g. The platelet pellet was then washed twice in resuspension buffer (Hepes-buffered Tyrode's solution, pH 6.5, containing 5 mM Hepes, 4 mM NaH$_2$PO$_4$, 137 mM NaCl, 2.6 mM KCl, 1 mM MgCl$_2$, 5 mM glucose, 1 mg/ml BSA) containing 5.0 U/ml apyrase and centrifuged at room temperature for 20 min at 3,000 g and resuspended to a final concentration of 10^9 platelets/ml in platelet buffer (5.0 mM Hepes, pH 7.3, containing 140 mM NaCl and 1 mg/ml BSA).

Platelet ultrastructural studies. Washed platelets either unactivated or activated for 3 min with 1 U/ml thrombin were centrifuged gently at 3,000 g for 5 min and the pellets fixed by addition of 1 ml of 0.1 M phosphate buffer, pH 7.4, containing 4% formaldehyde and 1% glutaraldehyde, postfixed in OsO$_4$, dehydrated in graded ethanol, and embedded in Epon. Thin sections were prepared with an ultramicrotome (LKB III, LKB Instruments, Inc., Gaithersburg, MD), stained with methanolic uranyl acetate and lead citrate, and examined with a transmission electron microscope (model 10A, Carl Zeiss, Inc., Thornwood, NY).

Platelet adhesion assay. The number of adherent platelets was essentially determined as previously described (13). Briefly, 100 μl of 5 × 10^8 platelets/ml were added to microtiter plates, the wells of which were pretreated overnight at 4°C with 50 μl of a 40-μg/ml protein solution prepared in platelet buffer or in 5 mM acetic acid for rat skin collagen, and the wells were blocked for 1 h with 1% BSA. Platelets were incubated in the wells for 30 min and nonadherent platelets were removed by aspiration and wells washed three times with 200 μl of platelet buffer. The number of adherent platelets was determined by measuring the platelet-derived protein using the BCA protein assay. The number of adherent platelets was proportional to the platelet-derived protein as measured using the BCA protein assay.

Measurement of bound anti–TSP Fab fragments. To determine whether anti–TSP Fab fragments blocked platelet adhesion by binding immobilized TSP, bound anti–TSP Fab fragments were measured by a direct enzyme-linked immunoadsorbent assay (ELISA). Briefly, TSP-coated wells were treated for 16 h at 4°C with 50 μl of either a 600 μg/ml solution of anti–TSP Fab fragments or normal rabbit Fab fragments dissolved in platelet buffer, containing 2 mg/ml of BSA. Plates were gently rocked during the incubation time. Wells were then washed

three times with 200 μl of platelet buffer and treated with 50 μl of a 1:4,000 dilution of biotin-conjugated goat anti–rabbit Fab-specific IgG. Wells were washed as before and treated with 50 μl of a 0.20 U/ml solution of alkaline phosphatase–coupled avidin D in platelet buffer, incubated for 15 min at room temperature, washed as before, and treated with 50 μl of alkaline phosphatase substrate solution (1 mg/ml of p-nitrophenylphosphate in 0.10 M glycine, pH 10.4, containing 1 mM ZnCl$_2$ and 1 mM MgCl$_2$). After 30 min, color development was stopped by the addition of 5 μl of 1 N NaOH and absorbances were determined at 405 nm.

Measurement of TSP contaminants by direct ELISA. Microtiter platelets coated with either 2 μg of TSP or with 2 μg of various adhesive proteins were blocked with 1% BSA for 1 h. Wells were incubated for 1 h with 50 μl of various dilutions of the first antibody in 10 mM phosphate buffer, pH 7.4, containing 150 mM NaCl and 0.05% Tween-20 (PBS-T). Wells were washed three times in PBS-T and incubated for 1 h with 50 μl of a 1:800 dilution in PBS-T of either alkaline phosphatase-coupled goat anti–mouse or anti–rabbit IgG. Wells were washed three times with PBS-T followed with three washes of 10 mM phosphate buffer, pH 7.4, containing 150 mM NaCl (PBS). Wells were treated with 50 μl of alkaline phosphatase substrate solution and color developed as described in the previous section.

Measurement of serotonin release. Washed platelets (5 × 10^8 platelets/ml) were incubated for 20 min with 0.25 nmol/ml [^{3}H]serotonin (10.7 Ci/mmol sp act). 100-μl aliquots of serotonin-loaded platelets were added to microtiter plates coated with TSP. After 40 min of incubation, suspensions were treated with 25 μl of EDTA-formalin solution, containing 77 mM EDTA and 10% formalin, to stop secretion. Suspensions were then centrifuged for 3 min at 10,000 g and the supernatants were removed. Radioactivity in the supernatants was measured by liquid scintillation counting. A 100% value for [^{3}H]serotonin was obtained from the supernatant of platelets treated with 0.2% Triton X-100. Percent release was calculated as [^{3}H]serotonin in the supernatant of the test platelets divided by [^{3}H]serotonin in the supernatant of Triton-treated platelets × 100.

Measurement of platelet IgG binding. 100 μg of purified IgG was labeled with ^{125}I-labeled iodine using Iodobeads as previously described (14). Free iodide was removed from protein solutions by centrifugation through small Sephadex G25 columns equilibrated in platelet buffer as previously described (15). Binding assays were performed as previously described (16). Briefly, 200 μl of a platelet suspension in the presence and absence of 100 μM Mn^{+2} was incubated with 2 μg/ml labeled IgG (∼ 100,000 counts/min) for 30 min. Suspensions were then layered on 50 μl of silicone oil prepared by mixing 2 parts light specific gravity oil (DC 200) with 8 parts high specific gravity oil (DC 500) and centrifuged for 2 min at 10,000 g. Resulting platelet supernatants and pellets were counted.

Analysis of data. All determinations were done in triplicate and the data are represented graphically using the program Sigma Plot, Jandel Scientific, Corte Madera, CA. The error bars represent the standard deviation.

Results

Purity of TSP preparations. To rule out the presence of contaminants in our TSP preparations that could contribute to the adhesive activity of TSP, TSP preparations were examined by direct ELISA for the presence of fibronectin, vitronectin, and collagen (Table I). Consistent with our previous studies (6), no fibronectin, vitronectin, or collagen was detected.

Ultrastructural analysis of platelets. To confirm that the platelets used in the adhesion study were not activated, platelets were fixed and processed for transmission electron microscopy. Two groups of platelets were compared, those used in our adhesion studies and a control group activated with 1 U/ml of thrombin (Fig. 1). The electron micrographs in Fig. 1 showed

Table I. Direct ELISA Analysis of Purified TSP

Antibody	Substrates			
	TSP	Fibronectin	Vitronectin	Collagen
	Absorbance 405			
Control IgG	0.047	0.026	0.005	0.057
Anti–human TSP				
(MAb 054)	0.547	0.017	0.001	0.040
Anti–human				
fibronectin	0.020	0.658	0.001	ND
Anti–human				
vitronectin	0.013	0.025	0.686	0.040
Anti–human				
collagen IV	0.029	0.014	0.001	0.318

The ELISA procedure was performed as described in Methods. All antibodies were monoclonal. Primary antibodies were used at a concentration of 2 µg/ml. Results are the mean of duplicate determinations.

that the platelets used in the adhesion studies showed no evidence of activation such as shape change or degranulation. In contrast, when activated with 1 U/ml of thrombin platelets were degranulated and irregular in shape (Fig. 1 B). These results are consistent with the serotonin release studies and IgG binding studies described below indicating that the platelets used in this were not activated.

Divalent cation requirements for TSP-induced platelet adhesion. TSP-induced platelet adhesion was divalent cation dependent (Table II). Little or no platelet adhesion was observed in the presence of either buffer, or 1 mM EDTA. In contrast, platelet adhesion was observed in the presence of either Ca^{+2}, Mg^{+2}, or Mn^{+2}. Maximal potentiation of platelet adhesion was observed in the presence of either 100 µM Mn^{+2}, 1 mM Ca^{+2}, or 1 mM Mg^{+2}. Mn^{+2} gave the greatest potentiation, showing a 3- and 10-fold stimulation over that observed in the presence of either Ca^{+2} or Mg^{+2}, respectively. In contrast, platelet adhesion to fibrinogen was promoted to about the same extent by the three ions tested, whereas adhesion to fibronectin showed the same Mn^{+2} dependence as compared to TSP but revealed a higher Mg^{+2} sensitivity (Table II). It is unlikely the effect of Mn^{+2} on TSP-induced platelet adhesion was due to platelet activation or exposure of fibrinogen receptor sites but rather an increase in the affinity of the platelet receptor for TSP because of the following observations: (*a*) Mn^{+2} did not cause a significant increase in release of platelet serotonin as compared to buffer alone, whereas thrombin released 85% serotonin (Table III). (*b*) Mn^{+2} caused no increase over control buffer in platelet-bound IgG, anti–GPIIb-IIIa (AP-2), or anti–VLA-2 (6F1) (Table IV). (*c*) Mn^{+2} caused a < 30% potentiation of platelet adhesion to fibrinogen as compared to that observed in the presence of either Mg^{+2} or Ca^{+2}. If platelet activation and exposure of fibrinogen receptor sites were induced by Mn^{+2}, then one might expect to see more Mn^{+2}-potentiated fibrinogen-induced platelet adhesion than the 30% observed.

In summary, these results indicate that platelet adhesion to TSP is highly dependent on the presence and nature of divalent cations. The divalent cations Mn^{+2}, Mg^{+2}, and Ca^{+2} enhanced platelet adhesion to TSP by 84-, 9-, 25-fold, respectively, as compared to adhesion obtained in the absence of ions (2 mM
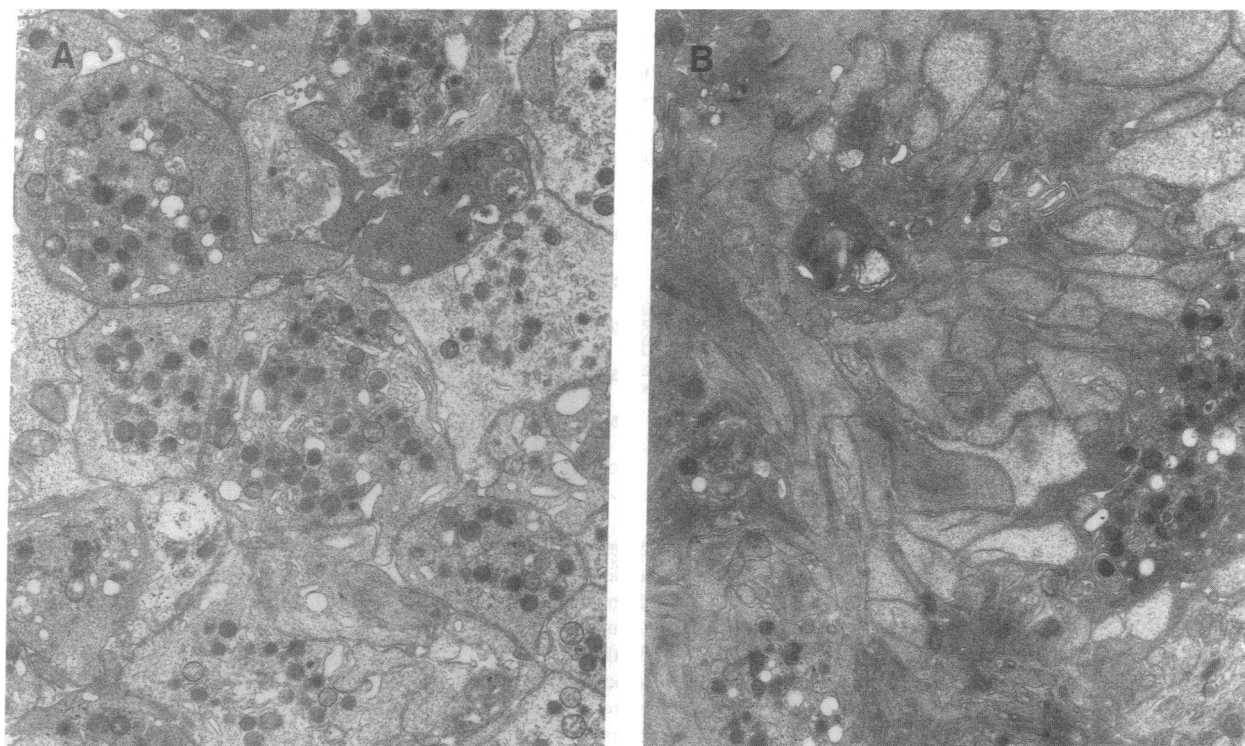

Figure 1. Transmission electron micrographs of the platelets used in the adhesion studies. (*A*) Platelets used in the adhesion studies. (*B*) Platelets activated with 1 U/ml thrombin. Platelets were processed for electron microscopy as described in Methods. *Note:* The platelets in *A* are discoid and granulated. In contrast, the platelets in *B* are clumped and irregular and the majority are devoid of granules. ×24,000.

Table II. Effect of Divalent Cations on Thrombospondin-, Fibrinogen-, and Fibronectin-induced Platelet Adhesion

Proteins	EDTA	Ca^{+2}	Mg^{+2}	Mn^{+2}
		Number of adherent platelets × 10^{-6}		
TSP	0.04±0.06	1.00±0.18	0.37±0.05	3.34±0.04
Fibronectin	0.11±0.02	1.25±0.26	1.70±0.08	3.40±0.09
Fibrinogen	0.36±0.03	1.45±0.13	1.65±0.10	2.10±0.03
Human serum albumin	0.00±0.02	0.00±0.02	0.10±0.02	0.20±0.03

100-μl platelet suspensions (5 × 10^8 platelets/ml) containing either 5 mM EDTA, 1 mM $CaCl_2$, 1 mM $MgCl_2$, or 100 μM $MnCl_2$ were added to microtiter plates, the wells of which were coated with either TSP, fibronectin, fibrinogen, or human serum albumin in platelet buffer and the wells blocked with 1% BSA. Platelets were incubated in the wells for 30 min and nonadherent platelets were removed by aspiration and wells washed three times with 200 μl of platelet buffer. The number of adherent platelets was determined by measuring the platelet-derived protein using the BCA protein assay as previously described (13). The data is presented as the mean of three replicates±SD.

EDTA). Since Mn^{+2} ion gave the greatest potentiation, all subsequent experiments were performed in the presence of 100 μM Mn^{+2}.

Effect of anti–TSP Fab fragments and an anti–TSP monoclonal antibody on TSP-induced platelet adhesion. To establish the specificity of TSP-induced platelet adhesion, immobilized TSP or fibrinogen, a protein that was previously found to constitute < 0.01% of our purified TSP preparations (5), were treated with anti–TSP Fab fragments or normal rabbit Fab fragments and evaluated for platelet adhesion and bound antibody (Fig. 2). The results of this experiment showed that anti–TSP Fab fragments specifically bound to TSP and inhibited platelet adhesion to TSP-coated surfaces. No inhibition of adhesion was observed with fibrinogen-coated wells. Similar results were obtained with an anti–TSP monoclonal antibody which completely inhibited adhesion to TSP, but had no effect on platelet adhesion to collagen (Fig. 3). These results indicate that platelet adhesion to TSP-coated wells is specific and not

Table III. Effect of Mn^{+2} on Platelet Serotonin Release

Incubation conditions	Secretion
	%
Buffer	9.3±0.5
$MnCl_2$ (100 μM)	11.6±0.7
Thrombin (1 U/ml)	85.0±5

Washed platelets (5 × 10^8 platelets/ml) were incubated for 20 min with [³H]serotonin. 100-μl aliquots of serotonin-loaded platelets were added to microtiter plates coated with TSP. After 30 min of incubation, suspensions were transferred to tubes containing 25 μl of EDTA-formalin solution to stop secretion. The suspensions were centrifuged for 3 min at 10,000 g and the radioactivity in the supernatants was measured. Percent release was calculated as [³H]serotonin in the supernatant of the test platelets divided by [³H]serotonin in the supernatant of Triton-treated platelets × 100. The data is presented as the mean of three replicates±SD.

Table IV. Effect of Mn^{+2} on the Binding of Mouse IgG, Antibody 6F1 (Anti–VLA-2), and Antibody AP-2 (Anti–GPIIb–IIIa) to Platelets

Ligand	Binding	
	Buffer	100 μM $MnCl_2$
	%	
Mouse IgG	3.2±0.2	2.8±0.5
6F1	16.1±0.5	17.7±0.4
AP-2	43.3±5.9	52.2±5.2

200 μl of platelet suspension in the presence and absence of 100 μM Mn^{+2} was incubated with 2 μg/ml labeled IgG ($\sim$ 100,000 counts/min) for 30 min. Suspensions were then layered on 50 μl of silicone oil and centrifuged for 2 min at 10,000 g. Resulting platelet supernatants and pellets were counted. Percent binding was calculated as the radioactivity in the pellet divided by the total radioactivity × 100. The data is presented as the mean of three replicates±SD.

mediated by possible contaminants, such as fibrinogen or collagen.

Effect of anti–platelet monoclonal antibodies and an RGD-containing peptide on TSP-induced platelet adhesion. To identify possible platelet receptors mediating TSP-induced platelet adhesion, platelets were incubated with various platelet monoclonal antibodies, and RGD-containing peptides and evaluated for their ability to adhere to TSP-coated microtiter wells (Table V). Maximum inhibition (60–89%) was obtained with monoclonal antibodies against VLA-2, GPIIb-IIIa, and with GRGDS. Inhibition with monoclonal antibodies against VLA-2 and GPIIb-IIIa was dose dependent (Fig. 4). However, anti–GPIIb-IIIa monoclonal antibodies gave not > 50–60% inhibition even at saturating concentrations (> 50 μg/ml) of antibody. These results suggest that platelet adhesion to TSP is partly mediated by GPIIb-IIIa, VLA-2, and other RGD-specific platelet adhesion receptors.
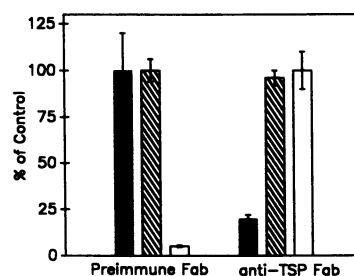

Figure 2. The effect of anti–TSP Fab fragments on TSP-induced platelet adhesion. Platelet suspensions (100 μl of 5 × 10^8 platelets/ml) were incubated for 30 min in the wells of a microtiter plate coated with either TSP or fibrinogen which had been previously treated with either preimmune Fab (designated preimmune Fab) or anti–TSP fragments (designated anti–TSP Fab) as described in Methods. The number of adherent platelets were determined by protein assay and the bound Fab fragments were determined from absorbance measurements by ELISA as described in Methods. The *solid bars* and *cross-hatched bars* represent platelet adhesion to TSP and fibrinogen, respectively. The *open bar graphs* represent the binding of Fab fragments to TSP-coated wells. Controls for platelet adhesion and Fab fragment binding were obtained from preimmune-treated wells and anti–TSP-treated wells, respectively. *Note:* Anti–TSP Fab fragments bound to TSP-coated wells, inhibited platelet adhesion to TSP-coated wells, but had no effect on platelet adhesion to fibrinogen-coated wells.
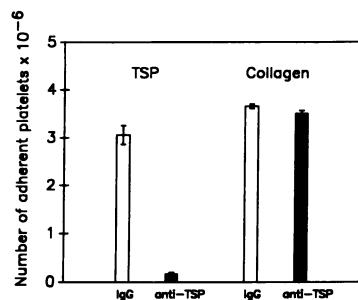
Figure 3. The effect of monoclonal anti–TSP on TSP-induced platelet adhesion. Platelet suspensions (100 μl of 5 × 10⁸ platelets/ml) were incubated for 30 min in the wells of a microtiter plate coated with either TSP or collagen and which had been previously treated for 1.5 h with either 50 μl of a 500-μg/ml mouse IgG solution in PBS containing 1% BSA (designated IgG) or 50 μl of a 500-μg/ml monoclonal anti–TSP antibody solution in PBS containing 1% BSA (designated anti–TSP). The number of adherent platelets were determined by protein assay as described in Methods. *Note:* Monoclonal anti–TSP inhibited platelet adhesion to TSP-coated wells, but had no effect on platelet adhesion to collagen-coated wells.

TSP-induced adhesion of normal and Glanzman's thrombasthenic platelets. To further establish that GPIIb-IIIa and VLA-2 are functioning as TSP adhesion receptors, TSP-induced adhesion of thrombasthenic platelets was studied. Platelets from two patients with Glanzman's thrombasthenia were obtained. These patients have been extensively studied by us (16) and others (17) and their platelets have been shown to aggregate only weakly in response to ADP or thrombin, bind < 10% of the fibrinogen of normal platelets, and contain < 10% of the GPIIb-IIIa found in normal platelets. We found that platelets from two thrombasthenic patients adhered to TSP 40–50% as well as normal platelets (Fig. 5). In addition, anti–GPIIb-IIIa (A₂A₉)-treated normal platelets (Fig. 5, *solid bars*) adhered to TSP approximately to the same extent as thrombasthenic platelets (Fig. 5, *open bars*) or anti–GPIIb-IIIa-treated thrombasthenic platelets (Fig. 5, *solid bars*). As expected since thrombasthenic platelets are deficient in GPIIb-

Table V. Effect of Monoclonal Antibodies and RGD Peptides on TSP-induced Platelet Adhesion

Antigen or peptide	Antibody	Inhibition
		%
GPIIb-IIIa	A₂A₉	60±3
GPIIb-IIIa	AP-2	50±10
GPIIIa	SSA6	0±2
GPIb	6D1	0±2
VLA-2 (GPIa-IIa)	6F1	82±12
VLA-2 (GPIa-IIa)	Gi9	68±16
GPIV	OKM5	0±3
VnR	LM609	4±2
GRGDS	—	89±4
GRGESP	—	0±3

100-μl platelet suspensions (5 × 10⁸ platelets/ml) containing 100 μM MnCl₂ and either 10 μg/ml antibody or 500 μg/ml peptide were added to microtiter plates, the wells of which were coated with TSP and blocked with 1% BSA. Platelets were incubated in the wells for 30 min and nonadherent platelets were removed by aspiration and wells washed three times with 200 μl of platelet buffer. The number of adherent platelets was determined by measuring the platelet-derived protein using the BCA protein assay as previously described (13). The data is presented as the mean of three replicates±SD.
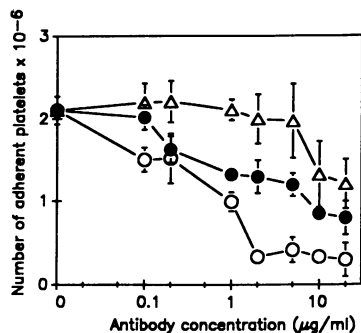

Figure 4. The effect of monoclonal antibodies against GPIIb-IIIa (AP-2) and VLA-2 (6F1 and Gi9) on TSP-induced platelet adhesion. Platelet suspensions (100 μl of 5 × 10⁸ platelets/ml) containing various concentrations of AP-2 (△), Gi9 (●), and 6F1 (○) were incubated for 30 min in TSP-coated microtiter wells. The number of adherent platelets were determined by protein assay. *Note:* Both 6F1 and Gi9 maximally inhibited adhesion by 70–90%, whereas AP-2 inhibited adhesion by < 50% at the highest concentration.

IIIa, anti–GPIIb-IIIa monoclonals had no effect on TSP-induced adhesion of thrombasthenic platelets.

Since two monoclonal antibodies against VLA-2 inhibited TSP-induced adhesion of normal platelets (Table V, Fig. 4), we assessed the effect of one of these antibodies (6F1) on TSP, FN, and collagen-induced adhesion of normal and thrombasthenic platelets (Fig. 6). Antibody 6F1 inhibited by > 90% TSP and collagen-induced adhesion of normal (Fig. 6, *open bars*) and thrombasthenic platelets (Fig. 6, *solid bars*). In contrast, 6F1 had no effect on adhesion of normal platelets to fibronectin, but inhibited fibronectin-dependent adhesion of thrombasthenic platelets.

These results support the conclusion that TSP-induced platelet adhesion is partly mediated through the platelet fibrinogen receptor–GPIIb-IIIa complex, and the putative platelet collagen receptor, VLA-2. Furthermore, since thrombasthenic platelet adhesion to fibronectin was also inhibited by anti–VLA-2, it appears that the VLA-2 complex functions as a fibronectin receptor in thrombasthenic platelets.

Discussion

TSP is a 450-D platelet-secreted protein composed of three identical disulfide-linked polypeptide chains (18). Under the electron microscope, the TSP polypeptide appears as long flexible chains with globular ends (19). TSP specifically interacts with a variety of macromolecules including heparin (20), fi-
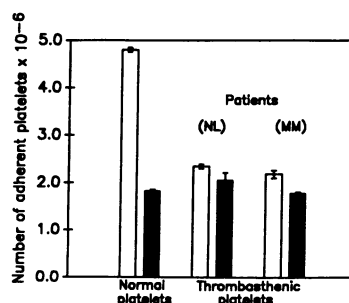

Figure 5. The effect of anti–GPIIb-IIIa monoclonal antibody (A₂A₉) on the TSP-induced adhesion of normal and thrombasthenic platelets. Normal and thrombasthenic platelet suspensions (100 μl of 5 × 10⁸ platelets/ml) were incubated for 30 min at room temperature in the absence (*open bars*) or the presence (*solid bars*) of 50 μg/ml of monoclonal anti–GPIIb-IIIa (A₂A₉) and then tested for TSP-induced adhesion. Patient initials are given in parentheses above the bar graphs. The number of adherent platelets were determined by protein assay. *Note:* Thrombasthenic platelets adhered 60% less well to TSP than normal platelets and anti–GPIIb-IIIa-treated normal platelets adhered to TSP to the same extent as thrombasthenic platelets.
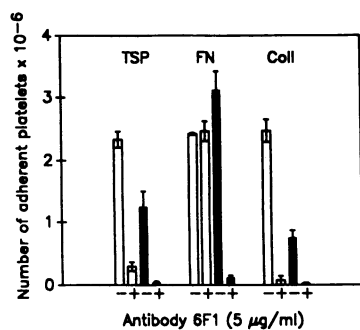
Figure 6. The effect of anti–VLA-2 monoclonal antibody (6F1) on TSP-, fibronectin-, and collagen-induced adhesion of normal and thrombasthenic platelets. Normal (*open bars*) and thrombasthenic (*solid bars*) platelet suspensions (100 μl of 5 × 10⁸ platelets/ml) were incubated for 30 min with antibody and evaluated for adhesion to either TSP (designated TSP), fibronectin (designated FN), or collagen (designated Coll). The number of adherent platelets were determined by protein assay. *Note:* 6F1 inhibited adhesion of thrombasthenic platelets to TSP, fibronectin, and collagen, whereas 6F1 inhibited adhesion of normal platelets to TSP and collagen but not to fibronectin.

brinogen (5), fibronectin (21), collagen (22), plasma proteins (23), and lipids (24). Like fibronectin, TSP possesses linear polypeptide domains that recognize heparin (25), fibrinogen (26), and collagen (27).

It is generally accepted that TSP plays a major role in mechanisms of cell–cell cohesion and cell–substratum adhesion. For example, TSP promotes platelet aggregation (4), platelet-monocyte interaction (28) and the cell substratum adhesion of a variety of cells including fibroblasts and endothelial cells (6), melanoma cells (29), squamous carcinoma cells (30), karatino-cytes (31), and osteoblasts (32). In addition, TSP has been shown to destabilize endothelial cell–fibronectin contacts and therefore modulate cell–substratum adhesion (33).

The studies described above establish a role for TSP in cell-substratum adhesion. However, it is not clear what role, if any, TSP plays in mechanisms of platelet adhesion. Previous studies have yielded conflicting results. For example, an early report by Lahav et al. (34) suggested a role for TSP in platelet adhesion. These investigators showed that TSP became cross-linked to either fibronectin or collagen, derivatized with a photoactivatable crosslinking agent, when platelets were allowed to adhere to these derivatized substrates immobilized on glass. Later, Lahav (35) published a report showing that glass and protein-covered surfaces when treated with TSP lose their capacity to bind unstimulated platelets. Similarly, Houdijk et al. (36) concluded that TSP played no role in subendothelial platelet interactions based on the observation that anti–TSP antibodies failed to block platelet adhesion to endothelial matrix when citrated blood was perfused over the matrix material under conditions of both low and high shear rate. In contrast, Adams and Hallee (37) demonstrated that TSP promoted platelet adhesion when red cells and platelets were perfused over TSP-covered plastic and glass surfaces at a shear rate of 100 s⁻¹ (37). Finally, Young et al. (38) clearly showed that platelets adhered and spread and fibrin was deposited on canine femoral arteriovenous shunts coated with human TSP and in contact with the circulating blood for 15 min.

The studies presented in this report support the hypothesis that TSP plays a role in cell–substratum adhesion. We observed that unactivated platelets specifically adhered to TSP-coated surfaces. The adhesion was divalent cation specific.

Mn⁺² ion was the most effective in promoting TSP-induced platelet adhesion. A similar Mn⁺² effect on TSP-induced cell adhesion has recently been reported for a variety of tumor cells including human melanoma cells (39). Other divalent ions tested, Ca⁺² and Mg⁺², promoted platelet adhesion but to a much lesser extent than Mn⁺². Fibronectin-induced platelet adhesion showed a similar ion dependence to that of TSP except that both Ca⁺² and Mg⁺² promoted more adhesion to fibronectin. In contrast, all three ions tested promoted approximately the amount of platelet adhesion to fibrinogen. These results suggest that platelet adhesion to various platelet adhesive proteins proceed by mechanisms requiring different ion specificities. Since the serum concentrations of Mn⁺² have been reported to be in the range of 0.154–0.736 μM (40), it is possible that Mn⁺² may promote the Ca⁺²- and Mg⁺²-dependent TSP-induced platelet adhesion in blood. Failure of Lahav (35) and Houdijk et al. (36) to observe significant TSP-dependent platelet adhesion could be due to the low affinities of platelets for TSP in the presence of Ca⁺² and Mg⁺² and Houdijk's use of citrated blood which would be expected to reduce the divalent ion concentration required to promote TSP-dependent platelet adhesion. The observation by Santoro (41) that only activated platelets adhere to TSP is difficult to reconcile with our observations since activated platelets in our experience adhere nonspecifically to BSA-coated surfaces. Furthermore, unactivated platelets would be expected to adhere to TSP, since Wolf et al. (42) found that unactivated platelets possessed 3,100 binding sites for TSP with affinities five times more favorable than for activated platelets.

The mechanism of Mn⁺²-promoted TSP-induced platelet adhesion most likely proceeds through an RGD-dependent platelet integrin. Mn⁺² probably promotes TSP-induced adhesion by increasing the affinity of the receptor for TSP, since Mn⁺² has been shown to enhance the binding of the fibronectin receptor to fibronectin 3–10-fold as compared to Ca⁺² and Mg⁺² (43). An alternative explanation based on the possibility that Mn⁺² is promoting TSP-induced platelet adhesion by activating platelets was ruled out since we could demonstrate that Mn⁺² did not stimulate generalized platelet activation, as measured by serotonin release, or exposure of TSP receptor sites as measured by binding of ¹²⁵I-labeled anti–GPIIb-IIIa or anti–VLA-2.

The identity of the platelet receptor mediating TSP-dependent platelet-adhesive interactions was of great interest to us because we had previously speculated on the possibility that GPIIb-IIIa functions as a TSP platelet receptor based on our observation that TSP binds GPIIb-IIIa in vitro (14). The specific functional consequence of this binding interaction, however, remains unclear since GPIIb-IIIa has been shown to interact with a number of platelet adhesive proteins, notably fibrinogen (44). In addition, several TSP-binding proteins have been reported in the literature which function in some capacity as either mediators of cell cohesion or cell adhesion. For example, endothelial cells (45) and melanoma cells (46) have a vitronectin-like receptor that functions in TSP-induced adhesion. Platelets also possess the same vitronectin-like receptor which binds TSP in vitro (47) but the function of this receptor in the platelet system is not yet known. Finally, platelet GPIV has been called the platelet TSP receptor because GPIV binds TSP in vitro (48) and mediates platelet–monocyte interaction (28), which is TSP dependent.

To identify the TSP adhesion receptor in our system, we assessed the antiadhesive activity of a number of monoclonal antibodies specific against platelet glycoproteins likely to function as TSP binding proteins. These antibodies were specific against GPIb, GPIV, the vitronectin receptor, GPIIIa, GPIIb-IIIa, and VLA-2. Only antibodies against platelet integrins, GPIIb-IIIa and VLA-2, blocked TSP-induced platelet adhesion. Anti–GPIIb-IIIa antibodies blocked 50–60% adhesion at saturating antibody concentrations, whereas anti–VLA-2 antibodies showed nearly total inhibition at antibody concentrations as low as 5–10 μg/ml, suggesting that both GPIIb-IIIa and VLA-2 mediate TSP-induced platelet adhesion. This conclusion was further supported by the observation that thrombasthenic platelets, which are essentially deficient in GPIIb-IIIa, adhered to TSP to the same extent as anti–GPIIb-IIIa-treated normal platelets, which have their GPIIb-IIIa membrane sites blocked with antibody and behave functionally like thrombasthenic platelets. Furthermore, in contrast to anti–GPIIb-IIIa, anti–VLA-2 monoclonal antibodies totally blocked TSP-induced adhesion of normal and thrombasthenic platelets. Anti–VLA-2 antibodies also blocked collagen-induced adhesion of normal and thrombasthenic platelets, suggesting that collagen and TSP share the same receptor on normal and thrombasthenic platelets. Interestingly, anti–VLA-2 had no effect on fibronectin-induced adhesion of normal platelets but inhibited fibronectin adhesion of thrombasthenic platelets. A possible explanation for this observation might be that thrombasthenic platelets which lack GPIIb-IIIa, a protein that functions both as fibrinogen and fibronectin receptor in platelets (49), are able to use VLA-2 as their fibronectin receptor. Taken together, these data suggest that both GPIIb-IIIa and VLA-2 function as receptors for TSP-induced platelet adhesion.

Finally, our data raise the possibility that TSP may not only play a direct role in platelet adhesion but also participate in platelet–collagen interactions. Our data may help to explain the hemorrhagic diathesis of a patient that suffered a bleeding tendency, characterized by frequent petechial bleeding, postoperative hemorrhages, and severe menstrual bleeding episodes, necessitating blood transfusions (2). The patient had a long bleeding time, abnormal collagen-induced adhesion, and defective collagen-induced aggregation, but normal ADP-induced aggregation. Analysis of her platelets revealed the absence of VLA-2 and intact TSP. TSP corrected the aggregation defect and, when TSP and VLA-2 appeared in her platelets after menopause, her bleeding tendency disappeared. One explanation for these patient data, as supported by the results of this study, is that TSP can bind either GPIIb-IIIa or VLA-2 and promote collagen-induced aggregation and adhesion by providing a platelet collagen binding site, since TSP interacts strongly with collagen (22). These data raise the fascinating possibility that TSP may function in blood to promote platelet collagen adhesion at low sheer rate while von Willebrand factor is active at high sheer rates.

Acknowledgments

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