Role of Thrombospondin in Platelet Aggregation

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Abstract. Thrombospondin (TSP), the major alpha-granule protein of human platelets, binds to the activated platelet surface upon platelet stimulation. TSP has hemagglutinating (lectin-like) activity and forms a specific complex with fibrinogen. Based on these observations, it was postulated that the interaction of TSP and fibrinogen on the activated platelet surface may be an important step in the platelet aggregation process. To test this hypothesis, monospecific, affinity-purified anti-TSP Fab fragments were prepared and their effects on platelet aggregation and platelet fibrinogen binding were studied. Anti-TSP Fab caused significant interference with thrombin- and collagen-induced platelet aggregation, as monitored by both turbidometric aggregometry and particle counting measuring the disappearance of single platelets. Phase-contrast microscopy revealed that anti-TSP Fab caused a marked decrease in platelet macroaggregates and an increase in microaggregates and nonaggregated single platelets. Anti-TSP Fab did not affect the initial phase of ADP-induced platelet aggregation but caused rapid platelet disaggregation with the abolition of the secondary phase of aggregation. The effect of anti-TSP Fab was not mediated by a direct inhibition of platelet secretion. The effect of anti-TSP Fab on specific binding of labeled fibrinogen to thrombin-stimulated platelets was also studied. Anti-TSP Fab caused a marked decrease in the affinity of fibrinogen binding to the receptors on the activated platelet surface. Kinetic analyses revealed significant displacement of labeled fibrinogen by unlabeled fibrinogen in the presence of anti-TSP Fab, suggesting that TSP serves to stabilize fibrinogen binding to the activated platelet surface and reinforces the strength of interplatelet interactions. It is proposed that platelet aggregation is a dynamic, multistep process, governed initially by the platelet membrane glycoprotein IIb/IIIa-fibrinogen interaction, with the TSP-fibrinogen interaction playing an important role in determining the size and reversibility of platelet aggregates.

Introduction

Thrombospondin (TSP)[1] is a major glycoprotein located in the alpha-granules of human platelets (1–6). Upon thrombin stimulation, TSP is secreted from the alpha-granules and binds to the activated platelet surface in the presence of Ca^{++} (7). Recent studies strongly suggest that the specific binding of fibrinogen to the platelet membrane glycoprotein IIb/IIIa complex plays a crucial role in the platelet aggregation process (8–15). Additional studies have demonstrated that fibrinogen specifically interacts with TSP (16, 17) and that the binding sites of fibrinogen for TSP are different from the binding sites for the platelet membrane glycoprotein IIb/IIIa complex (16). Furthermore, TSP has potent hemagglutinating activity, suggesting that TSP may be similar to the previously described thrombin-induced platelet lectin activity (18–21). Based on these observations, it was postulated that the interaction of fibrinogen and TSP on the activated platelet surface may be an important step in the platelet aggregation process (16). To test this hypothesis, monospecific anti-TSP Fab fragments were prepared and their effects on platelet aggregation and platelet fibrinogen binding were studied. The results suggest that TSP stabilizes the platelet aggregates and supports the conversion of reversible microaggregates to irreversible macroaggregates.

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1. *Abbreviations used in this paper:* Fg, fibrinogen; K_1, association rate constant; K_2, dissociation rate constant; PPACK, D-phe-pro-arg-chloromethyl ketone; PRP, platelet-rich plasma; TSP, thrombospondin; VWF, von Willebrand factor.

Methods

Materials. Human fibrinogen was purchased from MICO Corp. Ltd. (New York). Sepharose 2B, 4B, heparin-Sepharose CL-6B and protein A-Sepharose CL-4B were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). ADP and indomethacin were purchased from Sigma Chemical Co. (St. Louis, MO). Collagen was obtained from Hormon Chemie GmbH (Munchen, West Germany). Human alpha-thrombin (7,120 NIH U/ml) was a kind gift of Dr. John Fenton II (New York State Department of Health, Albany, NY). D-Phenylalanyl-prolyl-arginyl chloromethyl ketone (PPACK) was purchased from Calbiochem-Behring Corp. (La Jolla, CA). Carrier-free [^{125}I]sodium iodide and [^{14}C]serotonin were purchased from Amersham Corp. (Arlington Heights, IL) and New England Nuclear (Boston, MA), respectively. A dual channel platelet aggregometer was purchased from Payton Associates Inc. (Buffalo, NY). All other reagents used were of analytical grade.

Antisera. TSP was purified from thrombin-induced platelet releasate by Sepharose 4B gel exclusion chromatography and heparin-Sepharose affinity chromatography by the method of Lawler et al. (3) as previously described (16). Antisera to purified TSP were raised in rabbits by multiple intradermal injections of the protein in complete Freund's adjuvant. The anti-TSP sera were absorbed with insolubilized human fibrinogen. IgG fractions of the absorbed anti-TSP sera, as well as the pre-immune rabbit sera, were prepared as previously described (16). Fab fragments were prepared by papain digestion of the IgG fractions according to the method of Porter (22) and separated from the Fc fragments with a protein A-sepharose column. For immunopurification of anti-TSP Fab, purified TSP was coupled to CNBr-activated Sepharose 4B, and anti-TSP Fab was eluted from the TSP-Sepharose column with 0.1 M glycine, pH 2.5. The affinity-purified anti-TSP Fab formed a single precipitin arc with a goat-anti-rabbit F(ab')$_2$ serum and did not react with a goat-anti-rabbit Fc serum by double immunodiffusion. Anti-TSP reacted with TSP and did not show any specific interactions with other proteins in solubilized whole platelets by the Western blot technique (23). Anti-TSP Fab did not react with purified human albumin, fibrinogen, plasminogen, Factor VIII:von Willebrand factor (VWF) and PF-4 by enzyme-linked immunosorbent assay as detailed previously (16). PF-4 was prepared from thrombin-induced platelet releasate by means of heparin-Sepharose affinity chromatography as described by Handin and Cohen (24) and was homogeneous by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

Fibronectin (FN), prepared from human plasma by use of gelatin-Sepharose affinity chromatography followed by DEAE ion-exchange chromatography (25) and homogeneous by SDS-polyacrylamide gel electrophoresis, was a gift of Dr. Dominic Falcone (Cornell University Medical College). Anti-FN sera were raised in rabbit and monospecific, affinity-purified anti-FN Fab fragments were prepared in a similar fashion as anti-TSP Fab. Normal rabbit Fab fragments were purchased from Cappel Laboratories (Cochranville, PA).

Platelet aggregation studies. Sepharose 2B gel-filtered platelets were used for thrombin-induced platelet aggregation studies, and platelet-rich plasma (PRP) was used when ADP and collagen were used as agonists. Platelet aggregation was monitored turbidometrically by a dual channel aggregometer. In some studies, platelet aggregate size was estimated by phase-contrast microscopy.

Platelet aggregation was also measured using the particle counting technique of Frojmovic et al. (26) by counting the disappearance of single platelets from aggregating platelet suspensions. 0.4 ml of PRP was placed in a siliconized cuvette and stirred at 900 rpm. Nonimmune Fab or anti-TSP Fab was added. Platelet aggregation was initiated by the addition of collagen and monitored with the aggregometer. When optimal aggregation was reached, an equal volume of 2% glutaraldehyde was added directly to the cuvette. After fixation for 3 min, the fixed platelets were diluted fivefold with Tyrode's buffer and counted with a Coulter counter (Coulter Electronics Inc., Hialeah, FL). Counts were also made in duplicate by the use of a hemocytometer for 300–500 particles. The percentage of platelets aggregated, PA, was calculated from the following equation (26): $\%PA = (1 - N_t/N_0) \times 100\%$, where N_t and N_0 are, respectively, the nonaggregated platelet counts at times t and zero. An analysis of the size distribution of platelet aggregates containing two to seven platelets per aggregate were made in some of the above samples by direct examination with phase-contrast microscopy by use of a hemocytometer. The percentage of platelets in aggregates containing more than seven platelets per aggregate (macroaggregates) were obtained as follows: [100% − percentage of platelets in aggregates containing 1–7 platelets per aggregate] (26).

Platelet fibrinogen binding studies. Thrombin-induced platelet fibrinogen binding studies were performed according to the method of Hawiger et al. with slight modifications (27). Platelets were separated from plasma proteins by Sepharose 2B gel filtration of citrated PRP in divalent ion free Tyrode's buffer, pH 7.4, containing 0.35% bovine serum albumin (BSA). Purified human fibrinogen was radiolabeled with ^{125}I by the modified chloramine-T method (28). This preparation was 95% clottable with thrombin. Binding of ^{125}I-fibrinogen to gel-filtered platelets was carried out in 0.5 ml reaction mixtures containing 0.5×10^8 platelets in Tyrode's-BSA buffer with 0.5 mM $CaCl_2$. Platelets were stimulated with 0.1 U/ml thrombin with no stirring for 3 min. The thrombin activity was then inhibited by the addition of a thrombin specific inhibitor, PPACK, at 10^{-6} M (29). This concentration of PPACK completely inhibited the thrombin enzymatic activity towards fibrinogen, as determined by thrombin time assays. After incubation with PPACK for 5 min, normal rabbit Fab or anti-TSP Fab was added and incubated for 10 min. This was followed by the addition of ^{125}I-fibrinogen (specific radioactivity of 4,000–5,000 cpm/μg). After 30 min of incubation, 0.4-ml aliquots were removed and layered on 0.5 ml silicone oil (a mixture of Dow Corning 550 and 556 silicone oils [Dow Corning Corp., Midland, MI], 6.7:3.3 vol/vol, with specific gravity 1.040 as determined by a pycnometer) and spun for 10 min in a table-top microfuge (Beckman Instruments, Fullerton, CA). After centrifugation, the tips of the centrifuge pellets were sliced off and counted for ^{125}I. Each assay was done in duplicate, and all the incubation steps were done at room temperature. Nonspecific fibrinogen binding was measured by performance of the binding assays either in the absence of thrombin stimulation or by the addition of a 15-fold excess of unlabeled fibrinogen together with the ^{125}I-fibrinogen. Nonspecific binding equaled 15–20% of total binding by both methods. ADP-induced ^{125}I-fibrinogen binding was carried out similarly except that ^{125}I-fibrinogen and Fab fragments were added before ADP. For the determination of the association rate constant (K_1), the rate of specific fibrinogen binding at a variety of fibrinogen concentrations was measured. The dissociation rate constant (K_2) was determined by measurement of the rate of displacement of labeled fibrinogen from the platelet by a 27-fold excess of unlabeled fibrinogen in the presence of either normal rabbit Fab or anti-TSP Fab.

Binding of ^{125}I-anti-TSP Fab to thrombin-stimulated platelets. Anti-TSP Fab and normal rabbit Fab were labeled by the modified chloramine-T method (28). Binding of ^{125}I-anti-TSP Fab (specific

radioactivity 9.84 × 10^4 cpm/μg) and ^{125}I-normal rabbit Fab (specific radioactivity 9.72 × 10^4 cpm/μg) to thrombin-stimulated platelets was performed in the presence of 1 mM Ca^{++} as described for labeled fibrinogen binding. Fab fragments were used at 10 μg/ml. For this experiment, PRP was incubated with aspirin (0.1 mM) before gel filtration in order to minimize platelet activation during the isolation procedure.

Platelet serotonin release reaction. [^{14}C]serotonin release by thrombin from gel-filtered platelets was performed as previously described (30).

Statistical analyses for this study were performed with the aid of a TI-59 calculator and a computer (Apple Computer Inc., Cupertino, CA). A computer program for nonlinear least squares curve fitting (Ligand) (31, 32), developed by P. Munson, A. DeLean, and D. Rodbard, and modified for the Apple II by M. H. Teicher, was obtained from Biomedical Computing Technology Information Center, Vanderbilt Medical Center (Nashville, TN).

Results

Characterization of affinity-purified monospecific anti-TSP Fab. Affinity-purified monospecific anti-TSP Fab fragments were prepared. The monospecificity of the antibody was confirmed by its nonreactivity with albumin, Factor VIII:VWF, fibrinogen, plasminogen, fibronectin, and PF-4 by enzyme-linked immunosorbent assay. To demonstrate that the anti-TSP Fab interacted with TSP on the activated platelet surface, studies of ^{125}I-anti-TSP Fab binding to thrombin-stimulated platelets were performed (Fig. 1). There was specific binding of the radiolabeled anti-TSP Fab to thrombin-activated platelets, and the extent of binding correlated with the thrombin concentration. Maximal binding of the antibody was achieved at ~0.5 U/ml thrombin stimulation. Control studies using ^{125}I-nonimmune rabbit Fab did not show any significant binding.

Partial inhibition of thrombin-induced platelet aggregation by anti-TSP Fab. The effect of anti-TSP Fab on platelet aggregation was investigated. Anti-TSP Fab partially inhibited thrombin-induced aggregation of gel-filtered platelets (Fig. 2 *A*). In addition to a decrease in the extent of platelet aggregation, the size of the platelet aggregates was also diminished in the presence of the antibody, as evidenced by the reduced amplitude of the aggregation tracing, as well as by direct visualization by use of phase-contrast microscopy. Control studies using nonimmune Fab and affinity-purified anti-FN Fab did not show any inhibition at similar concentrations. Since the expression of TSP depends upon platelet activation and secretion (7), a double-dose thrombin stimulation experiment was performed to optimize the interaction of anti-TSP Fab with TSP on the activated platelet surface (Fig. 2 *B*). Gel-filtered platelets were first stimulated with a subaggregating dose of thrombin, allowing the expression of TSP and its interaction with anti-TSP Fab. A second aggregating dose of thrombin was then added. Significant inhibition of platelet aggregation was demonstrated by the anti-TSP Fab.

Partial inhibition of collagen- and ADP-induced platelet aggregation by anti-TSP Fab. Anti-TSP Fab produced significant partial inhibition of platelet aggregation by collagen, an agonist whose platelet stimulatory effect depends upon platelet secretion (Fig. 3). A decrease in the size of the platelet aggregates by the anti-TSP Fab was also noted as evidenced

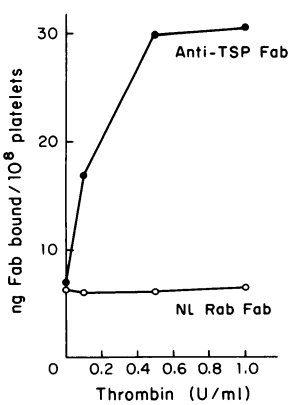

Figure 1. Binding of ^{125}I-anti-TSP Fab to thrombin-stimulated platelets. Suspensions of 5 × 10^7 gel-filtered platelets in Tyrode's-BSA buffer, pH 7.4, were incubated at room temperature with ^{125}I-anti-TSP Fab or ^{125}I-normal rabbit (NL Rab) Fab at 10 μg/ml final concentration. Thrombin at various concentrations was added and incubated without stirring for 3 min, and followed by the addition of PPACK. Platelet-bound ^{125}I-Fab fragments were determined by centrifugation of the platelets through silicone oil.

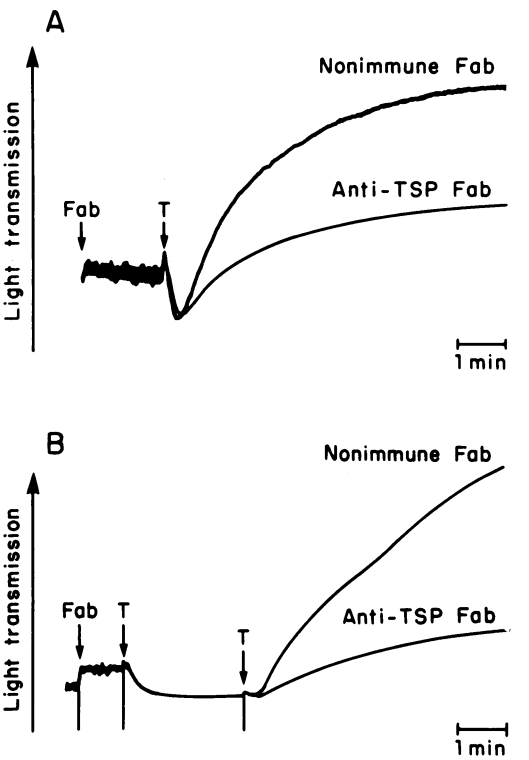

Figure 2. Effect of anti-TSP Fab on thrombin-induced platelet aggregation. (*A*) Gel-filtered platelets (2 × 10^8 platelets/ml) in Tyrode's-BSA buffer, pH 7.4, containing 0.5 mM $CaCl_2$, were incubated with anti-TSP Fab or nonimmune rabbit Fab at 70 μg/ml at 37°C. Platelet aggregation was initiated by the addition of thrombin (0.06 U/ml). (*B*) Experimental conditions were similar to those in *A* except that two different doses of thrombin were added. The first dose of thrombin was 0.026 U/ml and the second dose was 0.06 U/ml.

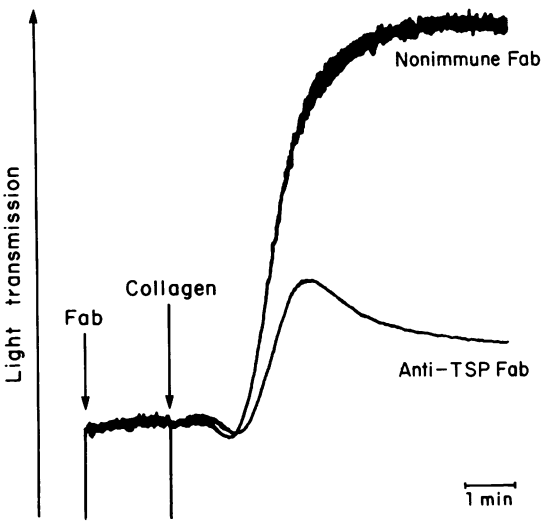

Figure 3. Effect of anti-TSP Fab on collagen-induced platelet aggregation. Citrated PRP was incubated with anti-TSP Fab or normal rabbit Fab at 100 μg/ml. Collagen was added at 0.88 μg/ml.

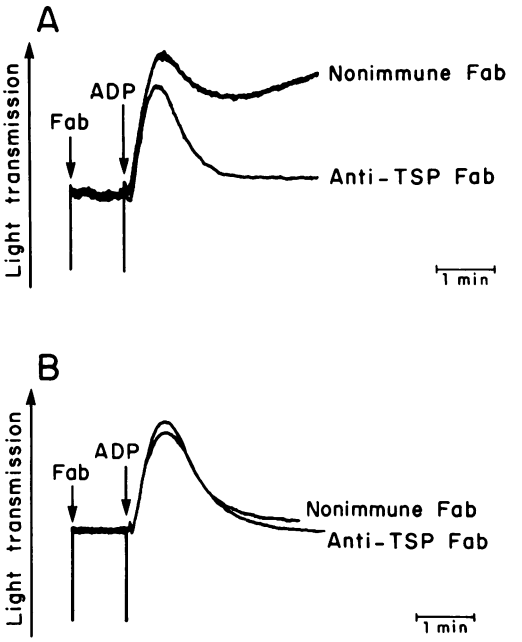

Figure 4. Effect of anti-TSP Fab on ADP-induced platelet aggregation. (*A*) Citrated PRP was incubated with anti-TSP Fab or control Fab at 100 μg/ml. ADP was added at 2 μM final concentration. (*B*) Citrated PRP was pre-incubated with indomethacin (30 μM final concentration) at 37°C for 20 min. Aggregation studies were then performed as described in *A*.

by reduced amplitude of the aggregation tracing as well as by direct examination by phase contrast microscopy. The presence of 5 mM EDTA completely abolished the aggregation response, indicating that the observed aggregometer tracings represented true collagen-induced platelet aggregation rather than platelet adhesion to polymerizing collagen fibrils.

The effect of anti-TSP Fab on ADP-induced platelet aggregation was also examined (Fig. 4 *A*). In the presence of anti-TSP Fab, the initial rate of platelet aggregation remained unchanged, with a slight decrease in the extent of the primary wave of platelet aggregation. The most striking effect of the anti-TSP Fab was the causation of rapid platelet disaggregation with the resultant abolition of the secondary wave of platelet aggregation. It has been previously demonstrated that ADP-induced platelet aggregation in citrated PRP is accompanied by significant platelet secretion (33). Our findings, therefore, suggested that the major inhibitory effect of anti-TSP Fab was not on the initial phase of platelet aggregation but on the secondary or secretion-dependent phase of platelet aggregation. To examine this possibility further, platelets were treated with indomethacin, which completely blocked ADP-induced platelet secretion (34). Anti-TSP Fab had no inhibitory effect on ADP-induced indomethacin treated platelet aggregation (Fig. 4 *B*), demonstrating that the anti-TSP inhibitory effect was directed mainly at the secretion-dependent phase of the platelet aggregation process.

Effect of anti-TSP Fab on platelet aggregation as determined by particle counting. Recent studies suggest that particle counting techniques for measuring platelet aggregation may be more sensitive than conventional turbidometric aggregometry (26). The effect of anti-TSP Fab on collagen-induced platelet aggregation was studied by both techniques (Table I). There was 80% inhibition of platelet aggregation as measured by planimetry of the aggregometry tracings and a 50% inhibition as determined from the disappearance of single platelets in the aggregating suspension; these results indicated a significant inhibitory effect of anti-TSP Fab on platelet aggregation as determined by both techniques. The nonaggregated platelet count was also determined with a hemocytometer, and results

Table I. Effect of Anti-TSP Fab on Collagen-induced Platelet Aggregation as Determined by Turbidometric Aggregometry and Particle Counting

	% Aggregation	
	Turbidometric aggregometry	Particle counting
Control Fab	100	100
Anti-TSP Fab	20±5	51.3±3

Collagen-induced platelet aggregation studies in the presence of control Fab or anti-TSP Fab were performed as described in Fig. 3. Turbidometric aggregometry results were determined by planimetry of the aggregation tracings. Particle-counting studies were performed on glutaraldehyde-fixed platelets from the same samples as in the turbidometric aggregometry, as described in Methods. Results were the mean (±SD) of three separate experiments.

were in good agreement with those obtained by the Coulter Counter. It is of note that the extent of platelet aggregation inhibition by anti-TSP Fab was greater as measured by turbidometric aggregometry. Phase-contrast microscopy revealed single, nonaggregated platelets and microaggregates (platelet aggregates ranging from doublets to those containing fewer than seven platelets per aggregate), with a marked decrease in the number of platelet macroaggregates (containing more than seven platelets per aggregate) in the presence of anti-TSP Fab, as compared with control with nonimmune Fab, where extensive macroaggregates were noted (Fig. 5).

Effect of anti-TSP Fab on platelet release reaction. To investigate the mechanism by which anti-TSP Fab interfered with the platelet aggregation process, the effect of anti-TSP Fab on thrombin-induced platelet release reaction was studied. Anti-TSP Fab did not cause any significant platelet release and did not interfere with the thrombin-induced platelet serotonin release (Table II).

Effect of anti-TSP Fab on thrombin-induced platelet fibrinogen binding. To determine whether the anti-TSP Fab inhibitory effect on platelet aggregation was mediated by interference with platelet fibrinogen binding, thrombin-induced platelet fibrinogen binding was studied. Anti-TSP Fab partially inhibited specific fibrinogen binding to thrombin-stimulated platelets (Fig. 6 *A*). Scatchard plot analysis of the equilibrium binding studies revealed that, in the presence of nonimmune Fab, thrombin stimulation exposed a uniform population of fibrinogen receptors, with an apparent dissociation constant (K_D) of 2.65×10^{-7} M, and ~57,800 fibrinogen molecules were bound per platelet (Fig. 6 *B*). This is in good agreement with previously published results (27). When ^{125}I-fibrinogen binding to thrombin-stimulated platelets was studied in the presence of anti-TSP Fab, there was a 4.2-fold increase in the K_D ($K_D = 1.12 \times 10^{-6}$ M), indicating a marked decrease in the affinity of fibrinogen binding to the platelet surface. There was also a slight decrease in the maximum number of fibrinogen molecules bound per platelet (52,500 fibrinogen molecules/platelet). When the data were analyzed by a computer program for nonlinear least squares curve fitting (Ligand) (31, 32), similar results were obtained: $K_D = 2.27 \times 10^{-7}$ M, with maximum binding sites of 55,900±5.1%/platelet for normal rabbit Fab; and $K_D = 1.02 \times 10^{-6}$ M, with maximum binding sites of 50,400±11%/platelet for anti-TSP Fab.

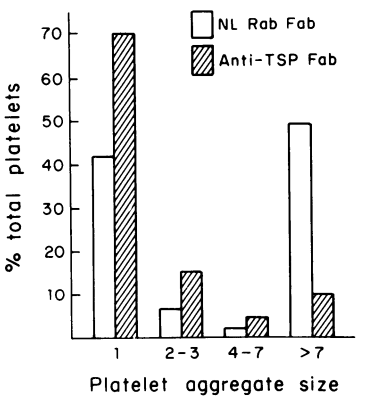

Figure 5. Effect of anti-TSP Fab on the size distribution of collagen-induced platelet aggregates. The size distribution of glutaraldehyde-fixed platelet aggregates from Table I was analyzed. Results were the mean of two separate experiments. NL Rab, normal rabbit.

Table II. Influence of Anti-TSP Fab on Platelet Serotonin Release

	% Serotonin release	
Thrombin	Anti-TSP Fab	Control Fab
U/ml		
0	0	0
0.05	27.5	26.5
0.10	42.3	46.0
1.0	60.7	56.6

Gel-filtered platelets were stimulated for 3 min with thrombin at 22°C in the presence of anti-TSP Fab or normal rabbit Fab at 100 μg/ml. [^{14}C]serotonin present in the supernatant after centrifugation at 11,750 rpm in a microfuge for 2 min (Beckman Instruments, Fullerton, CA) were quantified. Percent release was calculated relative to constituents solubilized by the addition of 1% Triton-X 100 to unstimulated platelets. Serotonin release was measured in the presence of 2 μM imipramine. Results are the average of two separate experiments, with duplicate determinations in each experiment.

Further control studies using an affinity-purified, monospecific anti-FN Fab at a similar concentration did not show any inhibition on thrombin-induced platelet fibrinogen binding (data not shown). The effect of anti-TSP Fab on ADP-induced platelet fibrinogen binding was also examined. In contrast to thrombin stimulation, anti-TSP Fab did not interfere with the specific binding of fibrinogen to ADP-stimulated platelets (Table III). Under the conditions employed in the fibrinogen binding assays, ADP stimulation did not cause any significant platelet release reaction (35).

Kinetic analyses of platelet fibrinogen binding in the presence of anti-TSP Fab. To characterize further the effect of anti-TSP Fab on thrombin-induced platelet fibrinogen binding, K_1 and K_2 of fibrinogen binding in the presence of anti-TSP Fab were determined. The value of the K_1 as determined by measurement of the rate of specific fibrinogen binding at different fibrinogen concentrations in the presence of nonimmune Fab was 0.17×10^6 M^{-1} min^{-1}. This is in good agreement with previously published K_1 values for ADP-induced fibrinogen binding (35, 36). Anti-TSP Fab did not cause any significant change in the K_1, which was determined to be 0.19×10^6 M^{-1} min^{-1}. The K_2 was determined by measurement of the rate of displacement of labeled fibrinogen from the platelets by an excess of unlabeled fibrinogen. In the presence of nonimmune Fab, there was minimal displacement of labeled fibrinogen by unlabeled fibrinogen (Fig. 7). When excess unlabeled fibrinogen was added together with anti-TSP Fab, there was significant displacement of labeled fibrinogen from the platelet surface, with a $t_{1/2}$ of 1

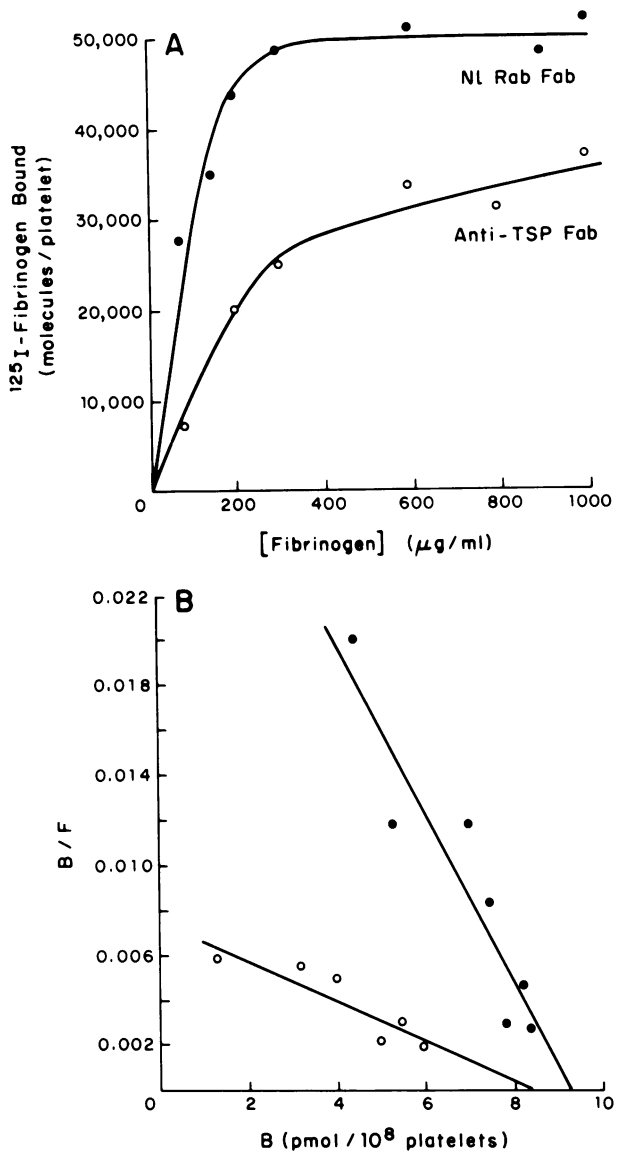

Figure 6. (*A*) Effect of anti-TSP Fab on ^{125}I-fibrinogen binding to thrombin-stimulated platelets. Suspensions of 5×10^7 gel-filtered platelets were incubated with thrombin (0.1 U/ml) with no stirring for 3 min. PPACK was added to inhibit thrombin activity. Anti-TSP Fab or control Fab (NL Rab, normal rabbit) (100 μg/ml final concentration) was added and incubated for 10 min. ^{125}I-Fibrinogen at increasing concentrations was added and incubated for 30 min. All incubation steps were done at room temperature. Specific fibrinogen binding was determined by centrifugation of the platelets through silicone oil. Each point is the mean of duplicate determinations. (*B*) Scatchard analysis of thrombin-stimulated ^{125}I-fibrinogen binding in the presence of anti-TSP Fab. The data from Fig. 6 *A* were analyzed. The lines represent linear regression by least squares of data points. Closed circles represent binding studies performed in the presence of control Fab ($r = -0.92$), and open circles represent binding studies performed in the presence of anti-TSP Fab ($r = -0.91$). B/F, bound/free.

Table III. Effect of Anti-TSP Fab on ADP-induced Platelet ^{125}I-Fibrinogen Binding

	Fibrinogen bound
	molecules/platelet
ADP + normal Rabbit Fab	17,400
ADP + Anti-TSP Fab	15,600

Final concentrations for ^{125}I-fibrinogen and Fab fragments were 150 and 100 μg/ml, respectively. ADP was added at 10 μM final concentration.

min and a K_2 of 0.693 min^{-1}. When the K_D was calculated from the reaction kinetics according to the formula $K_D = K_2/K_1$ (reference 37), K_D in the presence of anti-TSP Fab was equal to 3.65×10^{-6} M, in good agreement with the K_D determined by Scatchard plot analysis of the equilibrium binding studies (Fig. 6 *B*).

Discussion

The objective of the present study was to use the monospecific anti-TSP Fab in platelet aggregation and platelet fibrinogen binding studies to test the hypothesis that TSP may play an important role in the platelet aggregation process. Anti-TSP Fab caused significant interference with thrombin- and collagen-induced platelet aggregation, as monitored by both conventional turbidometric aggregometry (Figs. 2 and 3), and by particle counting measuring the disappearance of single platelets (Table II). The extent of platelet aggregation inhibition was apparently greater as measured by aggregometry (80% inhibition) than as determined by counting of nonaggregated platelets (50% inhi-

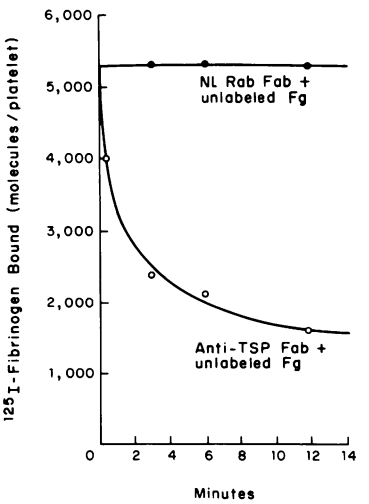

Figure 7. Displacement of platelet-bound ^{125}I-fibrinogen by anti-TSP Fab and unlabeled fibrinogen (Fg). Suspensions of 5×10^7 gel-filtered platelets were incubated with thrombin (0.1 U/ml) with no stirring for 3 min, and followed by the addition of PPACK. ^{125}I-Fibrinogen (50 μg/ml) was then added. After incubation for 10 min, a 27-fold excess of unlabeled fibrinogen together with either anti-TSP Fab or control Fab (100 μg/ml) was added, and dissociation of platelet-bound radioactivity was measured. NL Rab, normal rabbit.

bition). This agrees with recent studies showing that conventional aggregometry detects only the formation of platelet macroaggregates (>7 platelets/aggregate) and is insensitive in the detection of platelet microaggregates (platelet aggregates from 2 to 7 platelets/aggregate) (26). This was confirmed by phase-contrast microscopy which revealed that, in addition to causing an increase in nonaggregated single platelets, anti-TSP Fab caused a marked decrease in the number of platelet macroaggregates and an increase in the microaggregates (Fig. 5). Since both thrombin and collagen are strong inducers of platelet release, and TSP expression on the activated platelet surface depends upon platelet release (7), the effect of anti-TSP Fab on ADP-induced platelet aggregation was investigated. Anti-TSP Fab did not significantly affect the initial rate and extent of ADP-induced platelet aggregation but did cause rapid platelet disaggregation with the resultant abolition of the secondary wave of aggregation (Fig. 4 *A*). The lack of an inhibitory effect of anti-TSP Fab on the initial phase of platelet aggregation, before the release reaction, was further demonstrated by use of indomethacin-treated platelets in which the platelet release reaction was blocked. Anti-TSP Fab did not affect the aggregation response of these platelets to ADP stimulation (Fig. 4 *B*). Taken together, the platelet aggregation studies strongly suggest that the inhibitory effect of anti-TSP Fab was directed at the secretion-dependent phase of the platelet aggregation process and was manifested in a shift of the distribution of the size of the platelet aggregates from macroaggregates to microaggregates and nonaggregated single platelets, which appears to correlate with platelet disaggregation. The inhibitory effect of anti-TSP Fab was not mediated by a direct inhibition of platelet secretion (Table II).

To gain further insights into the mechanism of anti-TSP Fab inhibition of platelet aggregation, specific binding of labeled fibrinogen to thrombin-stimulated platelets was studied. In the presence of normal rabbit Fab, the apparent K_D of fibrinogen binding was determined to be 2.65×10^{-7} M, with ~57,800 binding sites per platelet. The binding sites appear to exist as a single class of fibrinogen receptors on the thrombin-activated platelet surface. This is in good agreement with published studies (27, 38). In the presence of anti-TSP Fab, there was a 4.2-fold increase in the K_D of fibrinogen binding, indicating a marked decrease in the affinity of fibrinogen binding to the receptors on the activated platelet surface. The potential number of fibrinogen binding sites per platelet was also slightly decreased (10%), probably due to steric hindrance of the anti-TSP Fab on fibrinogen binding. Control studies showed that a monospecific, affinity-purified anti-FN Fab at a similar concentration did not inhibit platelet fibrinogen binding. Furthermore, anti-TSP Fab did not have any effect on ADP-induced platelet fibrinogen binding, where the platelet release reaction did not occur (Table III) (33). This is consistent with the platelet aggregometry results indicating that the anti-TSP inhibitory effect depends upon the platelet release reaction.

Kinetic analyses revealed that the increase in the K_D in the presence of anti-TSP Fab was due mainly to an increase in K_2 (Fig. 7), while K_1 remained essentially unchanged. The K_D as determined by K_2/K_1 was in good agreement with that determined by Scatchard plot analysis of the equilibrium binding studies. It is of note that there was no effective displacement of ^{125}I-fibrinogen from the thrombin-activated platelet surface by unlabeled fibrinogen in the presence of normal rabbit Fab, indicating that the radiolabeled fibrinogen had already become irreversibly bound within the first 10 min of incubation (Fig. 7). It has been previously shown that with ADP-stimulation, there appears to be a progressive stabilization of platelet-bound fibrinogen with time (36, 39). However, the apparent stabilization of platelet-bound fibrinogen in the present study, which occurred fairly rapidly, was probably not related to that observed with ADP stimulation, since the latter was independent of the platelet-release reaction (39). The significant displacement of ^{125}I-fibrinogen by unlabeled fibrinogen in the presence of anti-TSP Fab, with the resultant increase in the K_2 and K_D, strongly suggests that TSP plays an important role in the stabilization of fibrinogen binding to the activated platelet surface.

Recently it has been proposed that ADP-induced platelet aggregation involves the interaction of fibrinogen with its platelet receptor in a multi-step reaction, leading to the evolution of an initial reversible microaggregate state to an irreversible macroaggregate state (26, 39). We suggest that a similar sequence of events may occur in thrombin-induced platelet aggregation as illustrated by the following model:

$$P_R \xrightarrow{\text{thrombin}} P_A \overset{\text{Fg}}{\rightleftharpoons} \text{aggregation A} \xrightarrow{\text{TSP}} \text{aggregation B.}$$

Thrombin binding to its receptor on the resting platelet (P_R) leads to platelet activation (P_A). This is associated with the induction of the platelet fibrinogen receptor, which is mediated by the platelet membrane glycoprotein IIb/IIIa complex (8–15). Specific binding of fibrinogen to the glycoprotein IIb/IIIa complex, probably involving the fibrinogen D domain (40, 41), initiates the platelet aggregation process, with the symmetrical fibrinogen molecule serving as a bridge to crosslink the aggregating platelets. This primary phase of platelet aggregation represents the formation of microaggregates and reversible macroaggregates (aggregation A). Depending upon the intensity of the stimulus, this essentially reversible primary phase of platelet aggregation is followed by the secondary phase of aggregation (aggregation B), which depends upon TSP release from the alpha-granules and subsequent binding to the platelet surface (7). Our previous studies (16, 17), as well as the present findings, suggest that TSP, by interacting with fibrinogen at a site different from its glycoprotein IIb/IIIa binding site, serves to stabilize fibrinogen binding to the platelet surface and reinforces the strength of interplatelet interactions, thereby converting the reversible micro- and macroaggregates into an irreversible state. Platelet aggregation is perceived as a dynamic multi-step process, basically governed by the glycoprotein IIb/IIIa-fibrinogen interaction, with the TSP-fibrinogen interaction playing a major role in determining

the size and reversibility of the platelet aggregates. In this model, TSP does not exist as a separate fibrinogen receptor on the platelet surface, which is consistent with the finding of a single class of platelet fibrinogen receptors with thrombin stimulation. This model is also consistent with the observation that Glanzmann's thrombasthenic platelets, which are deficient in glycoproteins IIb and IIIa but have intact alpha-granule proteins including TSP, are completely defective in platelet aggregation (42). On the other hand, Gray platelets, which are deficient in alpha-granules, showed suboptimal aggregation response to thrombin and collagen (5, 6). Recently an altered form of platelet TSP has been identified in patients with essential thrombocythemia (43). In addition to TSP and fibrinogen, thrombin stimulation leads to specific expression and binding of Factor VIII:VWF, FN, and PF-4 on the activated platelet surface (44, 45). The above model does not preclude the participation of these alpha-granule proteins in the platelet aggregation stabilization process.

Recent studies suggest that TSP has multiple structural domains associated with independent functional activities. In addition to interacting with fibrinogen, TSP specifically interacts with heparin, fibronectin, collagen, histidine-rich glycoprotein, and plasminogen (3, 16, 17, 46–48). TSP has also been found in human endothelial cells, fibroblasts, smooth muscle cells, monocytes, and granular pneumocytes (49–54). It is therefore most likely that the stabilization role of TSP in the platelet aggregation process as reported in this study represents only one facet of the biology of this complex multifunctional molecule.

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