A model of platelet aggregation involving multiple interactions of Thrombospondin-1, Fibrinogen and GPIIbIIIa receptor.

Arnaud Bonnefoy*, Roy Hantgan‡, Chantal Legrand* and Mony M. Frojmovic†

*Unité 353 INSERM, Institut d'Hématologie, Université Paris VII, Hôpital St Louis, Paris, France, †Department of Physiology, McGill University, Montreal, Quebec, Canada, and ‡Department of Biochemistry, Wake Forest University School of Medicine, Winston-Salem, NC, USA.

Running title: Thrombospondin and platelet aggregation

Key words: fibrinogen, thrombospondin, GPIIbIIIa, latex beads, aggregation, aggregation efficiency, shear rate.

Address correspondence to:
Dr. M. M. Frojmovic,
Dept of Physiology,
McGill University
3655 Drummond, #1137,
Montreal, Quebec, Canada H3G 1Y6.
Phone: (514) 398-4326
FAX: (514) 398-7452
Email: mony@med.mcgill.ca
Acknowledgements: We gratefully thank Professors Theo van de Ven (Chemistry, McGill) and Harry Goldsmith (Medicine, McGill) for helpful discussions and suggestions; the Medical Research Council of Canada, the Heart and Stroke Foundation of Quebec (HSFQ) and the National Science Foundation (USA) for research support. Arnaud Bonnefoy was a recipient for salary support from Sanofi-Thrombose, the International Council for Canadian Studies, HSFQ and from the Société Française d’Hématologie, with travel money from the Quebec-France exchange program of FRSQ-INSERM, the latter supporting exchange between our two laboratories.
SUMMARY

Thrombospondin-1 (TSP) may, after secretion from platelet α granules, participate in platelet aggregation, but its mode of action is poorly understood. We evaluated the capacity of TSP to form inter-platelet cross-bridges through its interaction with Fg, using either Fg-coated beads or Fg bound to the activated GPIIbIIIa integrin (GPIIbIIIa*) immobilized on beads or on activated fixed platelets (AFP), i.e. in a system free of platelet signalling and secretion mechanisms. Aggregation at physiological shear rates (100-2000 s⁻¹) was studied in a microcouette device, and monitored by flow cytometry. Soluble TSP bound to and induced aggregation of Fg-coated beads dose dependently, which could be blocked by the amino-terminal heparin binding domain of TSP, TSP18. Soluble TSP did not bind to GPIIbIIIa*-beads or AFP, unless these were preincubated with Fg. The interaction of soluble TSP with Fg-GPIIbIIIa*-beads or Fg-AFP resulted in the formation of aggregates via Fg-TSP-Fg cross-bridges, as demonstrated in a system where direct cross-bridges mediated by GPIIbIIIa*-Fg on one particle and free GPIIbIIIa* on a second particle was blocked by the RGD mimetic Ro 44-9883. Soluble TSP increased the efficiency of Fg-mediated aggregation of AFP by 30-110% over all shear rates and GPIIbIIIa* occupancies evaluated. Surprisingly, TSP binding to Fg already bound to its GPIIbIIIa* receptor appears to block the ability of this occupied Fg to recognize another GPIIbIIIa* receptor, but this TSP can indeed cross-bridge to another Fg molecule on a second platelet. Finally, TSP-coated beads could directly co-aggregate at shear rates from 100 to 2000 s⁻¹. Our studies provide a model for the contribution of secreted TSP in reinforcing inter-platelet interactions in flowing blood, through direct Fg-TSP-Fg and TSP-TSP cross-bridges.
INTRODUCTION

Thrombospondin-1 (TSP) represents 20-30% of the glycoproteins stored in human platelet α-granules (1). Upon platelet activation and degranulation, TSP is released, and an important fraction is found associated with the platelet surface (2, 3). Several putative receptors and ligands for TSP at the surface of activated platelets have been described, including fibrinogen (Fg) (4-7), sulfatides (8) glycoprotein IV (GPIV, CD36) (9, 10) and integrin associated protein (IAP/CD47) (11). TSP may also interact with several integrins including αvβ3 and αIIbβ3 (GPIIbIIIa), through its cryptic Arg-Gly-Asp-Ala (RGDA) sequences (12). However the interaction of TSP with GPIIbIIIa is controversial (13-17).

The participation of TSP in platelet aggregation has been demonstrated by a variety of studies reporting inhibition of platelet aggregation and secretion, by anti-TSP antibodies (18-23) and synthetic or recombinant peptides of TSP (6, 24). Leung (1984) (19) suggested that the interaction of TSP with Fg on the surface of activated platelets stabilizes the binding of Fg to its receptor, the activated integrin GPIIbIIIa (GPIIbIIIa*), with only Fg participating in direct cross-bridges. Recent studies propose that TSP also interacts with the integrin associated protein (IAP/CD47) (11) and functions as a costimulator of platelet integrin GPIIbIIIa and GPIaIIa (25, 26). A direct role for TSP as a cross-linker of platelets involving TSP-Fg interactions was supported by studies performed with isolated platelet membranes or activated fixed platelets (AFP) bearing Fg (27, 28). However in these models, the correlation between numbers of ligands/receptors and kinetics/extent of aggregation was not addressed. Moreover, studies were generally performed under nearly static conditions (29) or in an aggregometer (27), i.e. non representative of the physiological flow environment and shear stresses.

In the present study, using well defined in flow-experimental models, free of plasma, red blood cells, platelet signalling and secretion, we isolated, measured and modelled i) the capacity of TSP to form and support inter-platelet cross-bridges through its interactions with Fg or with other TSP molecules, and ii) the contribution of TSP-mediated cross-bridges in the aggregation of platelets driven by Fg/GPIIbIIIa* interactions.
EXPERIMENTAL PROCEDURES

Reagents. Human platelet thrombospondin (TSP) was purified as published (30) and characterized by western blot for the absence of fibrinogen, von Willebrand factor and fibronectin. The recombinant protein TSP18, corresponding to amino acid residues 1-174 of human platelet TSP, was purified from inclusion bodies as previously described (6). Human fibrinogen (Fg), depleted of von Willebrand factor and fibronectin, was from Enzyme Research Laboratories (South Bend, IN). Purified human-platelet GPIIbIIIa receptor was isolated from human platelet membranes by lentil lectin affinity chromatography followed by Sephacryl S-300 HR gel filtration chromatography and eluted from the column with an HSC buffer (in mM: HEPES 5, NaCl 150, CaCl₂ 3, pH 7.4) containing 30 mM of β-OG (n-octyl-β-D-glucopyranoside) (31). All three proteins (TSP, Fg and GPIIbIIIa) appeared undegraded, with appropriate molecular weights, when analysed in reduced/unreduced forms by SDS-PAGE. Peptide Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) and fluorescein isothiocyanate (FITC) celite were from Calbiochem (La Jolla, CA). Ro 44-9883, a non-peptide analogue of the Arg-Gly-Asp (RGD) peptide but 1000 times more potent and selective for αIIbβ3 (GPIIbIIIa) than for αvβ3 (32), was kindly provided by Dr. T. Weller (F. Hoffmann-La Roche, Basel, Switzerland). Dr. T. Krais (Schering Co., Berlin, Germany) generously provided ZK 36 374, a stable prostacyclin analogue. Polystyrene latex beads (4.5 µm diameter) were from Polyscience (Warrington, PA), and surfactant-free aldehyde/sulfate polystyrene latex beads (4.5 µm diameter) were from Interfacial Dynamics Corporation (Portland, OR). FITC-labelled TSP (FITC-TSP), TSP18 (FITC-TSP18) or Fg (FITC-Fg) were prepared as previously described by Xia et al (33) for FITC labelling of Fg. FITC-Reopro was a gift from Centocor (Malvern, PA).

Preparation of Fg- or TSP-coated beads. Polystyrene latex beads were washed three times, at ~ 0.5% solids, and incubated with either 500 nM Fg in phosphate buffered saline (PBS), or 200 nM TSP in Tyrode buffer containing 2 mM Ca²⁺, at pH 7.4, for 30 min at room temperature (RT), and processed as previously-published for Fg (32). The beads were finally centrifuged and resuspended in distilled and deionised water (Fg-beads), or in Tyrode containing 2 mM Ca²⁺ (TSP-beads), at a concentration of 250,000 beads/µl, and stored at 4°C. Beads coated only with BSA (BSA-beads) following the same procedure served as controls in aggregation or protein binding studies. The number of Fg or TSP molecules bound to the beads was measured with FITC-labelled protein (diluted 1:10 with unlabelled
protein) as previously reported (34), with an average of 183,034 ± 11,740 Fg or 149,070 ± 26,942 TSP molecules per bead (i.e. 2882 ± 185 and 2347 ± 424 molecules per µm²), respectively. The FITC/molecule ratio was also used to determine the number of FITC-labelled molecules bound per bead or platelet in equilibrium binding studies.

*GRGDSP-activated GPIIbIIIa-beads (GPIIbIIIa*-beads)* were prepared with aldehyde/sulfate polystyrene latex beads as previously described (34) with 110 nM GPIIbIIIa and 1 mM GRGDSP at RT. Final beads were washed with BSA to block unoccupied sites, then washed and stored at 4°C in PBS, 61 µM Hepes, 0.1% BSA, pH 7.4 (200,000 beads/µl). The beads obtained had a total of 51,678 ± 4,935 GPIIbIIIa as measured by FITC-Reopro binding and 38,747 ± 7,948 GPIIbIIIa*, as measured by FITC-Fg binding at saturating concentrations (i.e. 610 ± 125 GPIIbIIIa* molecules per µm²).

*Washed platelets (WP)* were prepared from platelet rich plasma (PRP) by the "single centrifuging and dilution procedure" described by Goldsmith et al (35). Briefly, blood was taken from healthy volunteers, not on any medication, added into 3.8% sodium citrate (1:9 vol/vol blood), followed by centrifugation (150 x g, 15 min). PRP was removed and acidified to pH 6.5 with 0.1% citric acid and ZK 36 374 was added to 50 nM followed by centrifugation (800 x g for 15 min). The platelet pellet was gently re-dispersed and resuspended in Ca²⁺-free modified Tyrode buffer containing 0.35% (w/v) BSA, pH 7.4 (BAT buffer) and kept at 37°C.

*GPIIbIIIa-activated and fixed platelets (AFP)* were prepared according to Du et al (36) with modifications as previously reported (37). Briefly, PRP was diluted 10 fold with Ca²⁺-free BAT buffer, incubated at 37 °C for 5 min with 10 nM ZK 36 374, then for 5 min with CaCl₂ (1 mM), followed by 200 nM Ro 44-9883 to "activate" the GPIIbIIIa receptors for another 5 min (all at 37°C). The platelets were then fixed with freshly prepared 0.5% (w/v) paraformaldehyde, washed with BAT buffer, and stored at 4°C.

*Fg-GPIIbIIIa*-beads and Fg-AFP. GPIIbIIIa*-beads (10,000/µl in a total volume of 400 µl) were incubated with 150 nM Fg, in BAT buffer, 1 mM CaCl₂, for 30 min at RT. Beads were then incubated with 1 µM Ro 44-9883, for 30 min at RT, in order to displace reversibly-bound Fg from the beads, and block free GPIIbIIIa* to prevent...
aggregation due to GPIIbIIIa*-Fg-GPIIbIIIa* cross-bridges. In these conditions, 50% of bound Fg was irreversibly bound to GPIIbIIIa* receptors, as measured using FITC-Fg. After incubation, beads were pelleted (10,000 x g, 30 s), resuspended in 400 µl BAT buffer, 1 mM CaCl₂ containing 500 nM Ro 44-9883. Fg-AFP (40,000/µl in a total volume of 400 µl) were prepared using the same procedure, except that 500 nM Fg instead of 150 nM were used during the first incubation step. With AFP however, only ~ 5% of the surface bound Fg remained irreversibly attached after incubation of the platelets with Ro 44-9883. Fg-GPIIbIIIa*-beads and Fg-AFP were used for equilibrium binding and aggregation studies (see below).

Equilibrium binding studies.

All incubations were done at RT in the dark. Fg- or TSP-beads (10,000/µl) were incubated for 1 h with increasing concentrations of FITC-TSP or FITC-TSP18, in Tyrode buffer pH 7.4 supplemented with 1 mM CaCl₂, 1 mM MgCl₂, 1% (w/v) BSA and 0.05% (v/v) Tween 20 (modified Tyrode buffer). GPIIbIIIa*-beads or Fg-GPIIbIIIa*-beads (10,000/µl) were similarly incubated for 1 h with increasing concentrations of FITC-TSP but in modified Tyrode buffer. For effect of preincubation of TSP on GPIIbIIIa*-beads, the latter (10,000/µl) were incubated for 1 h in modified Tyrode buffer, with increasing concentrations of FITC-Fg (0 to 100 nM) preincubated 30 min with buffer or 80 nM TSP. Binding studies were done in parallel on albumin coated-beads (BSA-beads) used as a control for the non-specific binding and for calculation of $K_d$ and $B_{max}$.

Fg occupancy of GPIIbIIIa* on AFP and effects of TSP. We prepared AFP with Fg occupancy of GPIIbIIIa* at 3-6, 20 and 35%, by incubating AFP in BAT buffer, 1 mM CaCl₂, with 20 nM Fg for 2 min (3-6% occupancy) or 30 min (20%), or with 50 nM Fg for 30 min (35%), at RT, prior to shear. The occupancy was determined from the ratio of bound fluorescence to the maximal fluorescence obtained at saturating Fg concentration (33). To study the effect of TSP on Fg-mediated aggregation of AFP, Fg and TSP were preincubated 15 min at RT in a molar ratio of 1/4 (i.e. Fg/TSP = 20/80 nM or 50/200 nM), and AFP were incubated with this Fg/TSP mixture in the same conditions as for Fg alone. We found no changing in the specific FITC-Fg binding to AFP with TSP addition.

Effect of the addition of TSP on aggregation efficiency of GPIIbIIIa-beads decorated by Fg at very low receptor occupancy (0.5%). GPIIbIIIa*-beads (7000/µl) were incubated in BAT buffer, 1 mM CaCl₂, with 0.15 nM of FITC-
Fg for 30 min at RT to reach 0.5% receptor occupancy. Beads were then incubated 30 min at RT with buffer or 180 nM TSP, and sheared for 0-60 s at 300 s⁻¹. These studies were compared to beads prepared with 5% Fg-receptor occupancy which yield maximal kinetics of aggregation, as previously reported (34).

Aggregation in flow. Kinetics of aggregations of AFP or GPIIbIIIa*-beads and/or ligands (Fg, TSP) were determined in a microcouette, as previously described (34, 37). Briefly, suspensions (400 µl) were loaded in the gap between the two cylinders at a fixed shear rate (G). Shear was stopped at selected times and 20-µl subsamples taken, fixed with 0.8% (v/v) glutaraldehyde (5 vol.) and analysed by flow cytometry. The fraction of particles recruited into aggregates, PA (percentage of aggregation), was calculated by monitoring the decrease of single-bead particles number per unit volume. Aggregation efficiencies (α), defined as the fraction of all shear induced collisions that result in the formation of doublets (34), was determined from experimentally measured initial rate of beads or AFP removal into bead-bead or AFP-AFP doublet formation, as previously described (34, 37).

Aggregation of AFP during incubation with soluble Fg or Fg plus TSP was negligible, as confirmed by phase-contrast microscopy (Zeiss, 500x magnification). Moreover, we did not detect disappearance of platelets or beads from the samples due to adhesion to the walls of the microcouette during shear.

TSP-beads coaggregate during storage at 4°C to partially form doublets and triplets (with no significant higher multiplets) : ~23% and 13% respectively (mean of 15 measurements) of the total number of particles at time 0 of the shear. We corrected for this effect on calculated α by modifying our equations as previously described (34).

Data analysis. Data are expressed as mean ± SE (standard error of the mean). To fit the nonlinear equations to our data, we used a nonlinear regression curve-fitter software (Sigma Plot, Jandel Scientific Software, San Rafael, CA), as previously described (37).
RESULTS

Interactions of TSP and TSP18 with Fg-beads

Soluble FITC-TSP (200 nM) bound to immobilized Fg (Fg-beads) with a half-time of about 10 min and saturation by ~60 min of incubation at room temperature (RT) (Figure 1). Specificity of the binding was demonstrated with a recombinant NH$_2$-terminal domain of TSP, TSP18, as shown previously with Fg immobilized on microtiter wells (6), with up to 90% inhibition of FITC-TSP binding to Fg-beads at 2 µM, and 50% inhibition, IC$_{50}$, of about 550 nM (Figure 2). The adsorption isotherm curve of FITC-TSP binding to Fg-beads was distinctly biphasic, as seen by curves (1) and (2) in Figure 3.A (n=3). The $K_d$ value for the initial phase ([FITC-TSP]<150 nM (curve (1)) was 52±16 nM, corresponding to soluble TSP binding to immobilized Fg with high affinity (38, 39), with a maximum binding ($B_{max}$) of 4,172 ± 473 molecules. The second phase ([FITC-TSP]>200 nM) ($B_{max}$ of ~6,000 molecules) may correspond to a low affinity TSP to TSP binding as described previously (40) and explored further below. By comparison, FITC-TSP18 binding to Fg-beads was fitted with a one binding site model with a $K_d$ of 369±31 nM and a $B_{max}$ of ~190,000 TSP18 per bead (Figure 3.B). The difference of $B_{max}$ obtained with the entire TSP molecule as compared to the TSP18 fragment is explained by steric hindrances due to 1) the high density of Fg adsorbed on the beads (2882 Fg/µm$^2$, i.e. maximal density as previously published (34)), and 2) the fact that Fg is horizontally elongated when adsorbed on the beads (34). Under similar conditions, we did not detect any binding of soluble FITC-Fg to TSP-beads (results not shown).

Aggregation of Fg-beads by soluble TSP (Figure 4). In the absence of TSP, Fg-beads did not aggregate during 120 s of shear at 300 s$^{-1}$. However, Fg-beads preincubated for 20 min with 50 to 200 nM TSP before shear, aggregated dose dependently: aggregation efficiencies ($\alpha$), measured from initial rates of aggregation, increased from 5.3±0.1% to 14.0±2%, and the extent of aggregation at 120 s increased from 63±2% to 85±2%. For 200 nM of TSP, about 1-2% of all Fg at the surface of the beads was occupied by TSP (measured with FITC-TSP), corresponding to a surface density of ~29-58 TSP molecules/µm$^2$ (n=3). TSP18 (4 µM), added before starting the shear, inhibited TSP (200 nM)-induced Fg-beads aggregation ($\alpha$ decreased from 14.0±2% to 4.0±0.4%). However, TSP18 added only after 120 s of aggregation of Fg-beads by TSP (200 nM), could not dissociate the formed aggregates (Figure 4).
**Effect of TSP on Fg binding to GPIIbIIIa*-beads**

Soluble Fg competed with Fg-beads for the binding of FITC-TSP with an \(IC_{50}\) of ~ 240 nM (results not shown), indicating that TSP interacted with both soluble and immobilized Fg, as also shown previously (22). For this reason, we studied the influence of the preincubation of FITC-Fg with TSP on the affinity \((K_d)\) and the \(B_{\text{max}}\) of FITC-Fg binding to GPIIbIIIa*-beads. The isotherm curve of FITC-Fg binding to GPIIbIIIa*-beads was not modified when FITC-Fg was preincubated 30 min with 80 nM of TSP (Figure 5). In both conditions, FITC-Fg bound to GPIIbIIIa*-beads with a \(K_d\) of 23 ± 2.4 nM and a \(B_{\text{max}}\) of ~ 39,000 ± 8000 FITC-Fg molecules.

**Binding of FITC-TSP to GPIIbIIIa*-beads and Fg-GPIIbIIIa*-beads or platelets.** Soluble TSP did not bind to GPIIbIIIa*-beads (Figure 6). However, when GPIIbIIIa* receptors were decorated by irreversibly bound Fg molecules (Fg-GPIIbIIIa*-beads), soluble TSP bound to the beads in a saturable manner. The corresponding isotherm binding curve, fitted to the data using an equation for a one-binding site model, gave a \(K_d\) of ~ 23 nM. However the curve was best fitted by a two-binding site equation model, which gave two \(K_d\) values of ~ 5 and 163 nM, corresponding to \(B_{\text{max}}\) values of 2105 ± 178 and 4315 ± 806 molecules per bead, respectively. These observations were qualitatively confirmed for AFP and ADP (10 \(\mu\)M)-activated washed platelets, with no binding of FITC-TSP (100 nM, 45 min), in absence of platelet secretion verified by the lack of detectable surface expressed TSP. However, preincubation for 5 min with 100 nM Fg led to binding of FITC-TSP to the platelet surface (results not shown).

**Aggregation of Fg-GPIIbIIIa*-beads by TSP.**

We next determined the capacity for soluble TSP to form cross-bridges between receptor-bound Fg, in the absence of direct Fg cross-bridging to free GPIIbIIIa* receptors. For this experiment, GPIIbIIIa*-beads were first decorated by irreversibly bound Fg (Fg-GPIIbIIIa*-beads) and the free receptors were blocked by the RGD mimetic Ro 44-9883 (see inset in Figure 7). Thus, without TSP (Figure 7A), the aggregation of Fg-GPIIbIIIa*-beads was almost completely inhibited by Ro 44-9883 \((PA = 13.6 \pm 4.4\%, \text{ after } 120 \text{ s of shear rate at } 300 \text{ s}^{-1})\). However, in presence of 200 nM TSP, aggregation rapidly reached 70.1 ± 2.5% by 120 s with an efficiency of 18.1 ± 4.8%. In such conditions, about 10-15% of GPIIbIIIa*-bound Fg was occupied by TSP (calculated from Figure 6), corresponding to ~ 31-44 TSP molecules/\(\mu\)m\(^2\) (n=3), very similar to the density of TSP on Fg-beads mentioned above. We
reproduced the experiment using Fg bound to activated fixed platelets (Fg-AFP) instead of Fg-GPIIbIIIa*-beads (Figure 7B). As explained in the Experimental Procedures, only ~ 5% of surface-expressed activated GPIIbIIIa* on AFP remained occupied by Fg molecules in the presence of 1 µM Ro 44-9883. Thus, with all free GPIIbIIIa* blocked by Ro 44-9883, no aggregation of Fg-AFP occurred in the absence of TSP, but added TSP induced 12.5 ± 7.1% aggregation by 120 s of shear at 300 s⁻¹, with a related aggregation efficiency of 1.9 ± 0.2%. The TSP/Fg ratio measured at the surface of the platelets was the same as on Fg-GPIIbIIIa* beads (10-15%), corresponding here to a TSP surface density of 4-5 molecules/µm² (n=3).

**Effect of the addition of TSP on aggregation efficiency of Fg-GPIIbIIIa*-beads at low receptor occupancy (0.5%)**

Fg-GPIIbIIIa*-beads were prepared at low receptor occupancy (0.5%), in a range where aggregation efficiency varies rapidly with percent occupancy, previously reported to be between 0-20% for platelets (37) and 0-5% for our model beads (34). Incubation of these beads with 180 nM of TSP gave a partial decoration of bound Fg by TSP (10-15% of the bound Fg). In the presence of TSP, the kinetics of aggregation at 300 s⁻¹ was slowed down, corresponding to a decrease of α of ~30%, from 16.9 ± 0.1 % to 12.0 ± 1.2 % (figure 8).

**Effect of the addition of TSP on aggregation efficiency of AFP by Fg**

We first varied the Fg-receptor occupancy at a fixed shear rate of 300 s⁻¹. AFP were incubated with Fg or Fg plus TSP (molar ratio of 1:4) to yield Fg-GPIIbIIIa* occupancies of 3-6%, 20% and 35% with about 10-15% of Fg occupied by TSP. Aggregation efficiencies (α) for AFP sheared with Fg only, increased from 9 to 20% with increasing Fg receptor occupancy from 3-6% to 35% (Table 1). The addition of TSP induced a significant increase of α at all Fg-receptor occupancies tested (p<0.01 to p<0.07), with mean increases ranging from 30 to 61% (Table 1). We next varied the shear rate at a fixed receptor occupancy of 20%. Thus, AFP were incubated with Fg (20 nM) or Fg plus TSP (20/80 nM) for 30 min. In the absence of TSP, α decreased 15-fold when increasing the shear rate from 300 to 2000 s⁻¹. The addition of TSP induced a significant increase of α at both 300 and 2000 s⁻¹ (p<0.1) and an increase of 30% was also observed at 1000 s⁻¹ (p<0.3) (Table 2).

**Interactions of TSP with TSP-beads.** Soluble FITC-TSP could bind to TSP immobilized on polystyrene latex beads (TSP-beads) with a Kd of 732 ± 118 nM (Figure 9). Aggregate formation mediated by TSP-TSP interaction(s) was
investigated in flow by shearing TSP-beads from 100 to 2000 s\(^{-1}\) in the microcouette (Figure 10A). TSP-beads coaggregated at all shear rates tested. The extent of aggregation after 120 s of shear increased with increasing shear rate from 100 to 300 s\(^{-1}\) (59 ± 3% and 77 ± 3%, respectively), but decreased at higher shear rates (67 ± 0.4% and 49 ± 3% at 1000 and 2000 s\(^{-1}\), respectively). The calculated related \(\alpha\) indicated that the high efficiency of TSP-beads coaggregation at 100 s\(^{-1}\) (22.2 ± 4.9%) decreased by about 10-fold and 40-fold at 1000 and 2000 s\(^{-1}\), respectively (Figure 10B). When TSP-beads were sheared at 300 s\(^{-1}\) in the presence of 8 mM EDTA, both \(PA\) (Figure 10A) and \(\alpha\) were reduced by >80% and >90% respectively. TSP-beads did not aggregate either with Fg-beads or GPIIbIIIa*-beads (results not shown).
DISCUSSION

We studied the involvement of thombospondin-1 (TSP) in platelet aggregation through its interaction with fibrinogen (Fg) bound to its receptor, the activated GPIIbIIIa integrin (GPIIbIIIa*). We used model particles, either latex beads with surface-adsorbed Fg (Fg-beads) or Fg bound to surface-captured GPIIbIIIa* (Fg-GPIIbIIIa*-beads), or activated fixed platelets (AFP) bearing GPIIbIIIa* with bound Fg (Fg-AFP). These models were chosen to mimic the surface of activated platelets but in the absence of any signalling or secretion process, thereby isolating the cross-bridging functions of TSP while monitoring qualitative and quantitative surface expression of ligands.

Soluble TSP induced aggregation of Fg-beads dose-dependently, with a maximal effect observed at 200 nM, and an aggregation efficiency (α) of 14 ± 2.1% at a shear rate of 300 s⁻¹. Aggregation of GPIIbIIIa*-beads by TSP only occurred after preincubation of the beads with Fg. With added Ro 44-9883, the RGD mimetic, in order to block the cross-bridges between GPIIbIIIa* bound Fg and free GPIIbIIIa*, aggregation occurred through Fg-TSP-Fg cross-bridges with α = 18.1 ± 4.8% at 300 s⁻¹ similar to that obtained with Fg-beads at comparable TSP surface densities (~30-60 TSP per µm²). This experiment reproduced with AFP gave comparable results: aggregation by TSP at 300 s⁻¹, with added Ro 44-9883, only occurred in the presence of receptor-bound Fg, with α lower than seen for Fg-GPIIbIIIa*-beads, expected for the 10-fold lower density of Fg and TSP in this system. These experiments clearly demonstrate that TSP can induce aggregation of beads or AFP by directly cross-bridging two Fg presented on two particles, at a physiological shear rate (300 s⁻¹). A recombinant fragment encompassing residues 1-174 of TSP, TSP18, previously shown to inhibit the secretion-dependent phase of platelet aggregation (6), inhibited aggregation of Fg-beads by TSP but did not disaggregate the formed aggregates. This is an indication for 1) the involvement of the amino-terminal part of the TSP molecule in TSP-Fg interactions and 2) a strong TSP-Fg interaction (off-rate very low), as also observed in the binding of FITC-TSP to Fg-GPIIbIIIa*-beads, where TSP18 was not able to displace the bound FITC-TSP but inhibited any further binding of FITC-TSP (results not shown). There are several putative binding sites on Fg and TSP which could stabilize or reinforce their mutual interactions, including three sites on Fg: the Aα 241-476 (4), Aα 113-126 and Bβ 243-252 (5); and at least two sites on TSP: TSP 1-174 (6), potentially the same as TSP 151-164 (41), and TSP 385-522 (42).

Interestingly, preincubation of FITC-Fg with TSP did not modify the affinity (Kd) nor the maximum binding (Bmax) of FITC-Fg to GPIIbIIIa* immobilized on beads (Figure 5). This is in accord with the study of
Boukerche and McGregor (23) who showed that a MoAb anti TSP (P8) that inhibits platelet aggregation by low
doses of thrombin (0.05-0.06 U/ml) did not affect the dissociation constant of Fg binding to platelets stimulated with
ADP (10 µM) or thrombin (0.4 U/ml). However, Leung (19) postulated that TSP, by interacting with Fg at a site
different from its GPIIbIIIa* binding site, increases the affinity of Fg for GPIIbIIIa*, thereby stabilizing the
aggregates. This assumption, based on the observation that an anti-TSP Fab decreased the affinity of Fg binding to
thrombin activated platelets, could rather reflect a steric hampering caused by the Fab bound to TSP, that would
disable the closely located Fg/GPIIbIIIa* interactions described by several authors (9, 43, 44).

Surprisingly, our studies suggest that a receptor-bound Fg with attached TSP is no longer capable of
interacting with another GPIIbIIIa*, as incubation of Fg-GPIIbIIIa*-beads (0.5% of receptor occupancy) with TSP
decreases the aggregation efficiency by ~30%. In this experiment, we expect that Fg-bound TSP (~ 10-15% of
receptor-bound Fg contain a TSP molecule), cannot find a counterpart Fg molecule on an adjacent bead because of
the low surface density of Fg and TSP molecules (~200 Fg/bead and ~25 TSP/bead). This limitation is not
encountered by the Fg-GPIIbIIIa* cross-bridging system since ~39,000 GPIIbIIIa* are available for each Fg on
adjacent beads. Using a standard curve reporting the efficiency of GPIIbIIIa*-beads aggregation for varying Fg-
receptor occupancies, as previously published (34), we determined that the aggregation efficiency in the presence of
TSP is equivalent to the aggregation efficiency that would be obtained with ~15 % fewer functional Fg on
GPIIbIIIa*-beads, closely corresponding to the percentage of Fg occupied by TSP. This hypothesis may explain the
“anti-adhesive” property of TSP previously reported in the literature for platelet adhesion to immobilized Fg or
fibronectin, after preincubation of these supports with soluble TSP (45, 46). Our results support the idea that the anti-
adhesiveness of TSP is induced by the loss of the reactive site on Fg or fibronectin for its platelet receptor, following
its occupancy by TSP.

Nevertheless, when testing the effect of addition of TSP on the aggregation efficiency of AFP mediated by
Fg and GPIIbIIIa*, we found an increase of 30-110% at all Fg-receptor occupancies (3-35%) and all shear rates
(300-2000 s⁻¹) tested, with 10-15% of receptor-bound Fg decorated by TSP. We expect that TSP-Fg associations on
the cell surface would accelerate the kinetics of aggregation (i.e. aggregation efficiency) by 1) increasing the length
of the bridges which will both increase the surface area available for cross-bridging (see figure 11), and decrease the
actual distance required for cell-cell interactions, thereby increasing the collision frequency, 2) increasing the
number of bridges between the platelets, and 3) concentrating adhesive molecules in multivalent adhesive structures (clusters) that will favour firm platelet to platelet adhesion (see below).

In addition to $Fg$-$TSP$-$Fg$ interactions, we have shown that $TSP$-$TSP$ interactions can also participate in cross-bridging to drive particle aggregation at varying shear rates. The beads co-aggregated at all shear rates, with a 10-fold decrease in efficiency for a 10-fold increase in shear rate ($100$-$1000 \text{ s}^{-1}$), similar to the 5-fold decrease previously reported for $Fg$-mediated aggregation of ADP-activated platelets driven by GPIIbIIIa*-Fg cross-bridges (37). The maximal aggregation efficiency at $300 \text{ s}^{-1}$ ($10.4 \pm 1.2\%$) was only 2-3 times lower than for $Fg$-mediated aggregation of particles or platelets with surface-bound GPIIbIIIa* (34, 37 and this study).

Our work demonstrates the direct contribution of TSP in reinforcing the inter-platelet interactions in physiological flow conditions. The participation of TSP is thought to be maximal during the early phase of the platelet secretion process when high concentrations of TSP may accumulate at the contact of activated platelets with $Fg$ already bound to a significant number of GPIIbIIIa*. Expected TSP-mediated cross-bridges are modelled in Figure 12: The $\text{GPIIbIIIa}^*-\text{Fg}-\text{GPIIbIIIa}^*$ cross-bridges (Figure 12.A) will cohabitate with $\text{GPIIbIIIa}^*-\text{Fg}-\text{TSP}-\text{Fg}-\text{GPIIbIIIa}^*$ crossbridges (Figure 12.B), with possible participation of $TSP(n)$ interactions (Figure 12.C). Direct $TSP$ or $TSP(n)$ cross-bridges might also form, involving TSP ligands/receptors other than GPIIbIIIa*-bound $Fg$ (CD36 ?, CD47 ?). This latter model may appear controversial because platelets from patients with Glanzmann’s thrombasthenia (GT) which lack GPIIbIIIa, have been shown to express normal levels of TSP at the cell surface upon thrombin activation, with no aggregation detected in an aggregometer (23, 47). However, this apparent contradiction with our model may rather arise from the facts that 1) micro-aggregates of <10 platelets observed in one GT patient by phase contrast microscopy may not be detected by aggregometry (48), and 2) electron microscopy studies revealed that upon thrombin activation, TSP was not distributed normally on the platelet surface of one GT patient, with decreased size and number of TSP clusters (49). It is therefore conceivable that in absence of GPIIbIIIa (and membrane-bound $Fg$), TSP may not be in an “efficient clustered” conformation to support platelet cross-bridging. Our model of co-aggregation of TSP-beads is expected to circumvent these experimental artefacts as 1) our experimental setting detects micro-aggregates as small as doublets, and 2) we used TSP-beads with high TSP-surface density ($2347 \pm 424$ molecules per $\mu$m$^2$) that possibly mimic the concentrated TSP found in clusters.

Finally our model suggests that macromolecular TSP/$Fg$/GPIIbIIIa* associations could also form, with TSP involved in both inter- and intra-platelet cross-bridges (Figure 12.D). The physiological relevance of such
associations is supported by previous electron microscopy studies showing co-localisation of TSP, Fg, CD36 and GPIIbIIIa in clusters on the surface of activated platelets (43, 44, 50, 51).

A role for TSP has been reported in intra-platelet costimulatory signalling resulting in an enhanced affinity/avidity of GPIIbIIIa (25). We have additionally demonstrated that TSP also provides an extracellular amplification system of platelet aggregation via inter-platelet cross-bridges possibly involving several molecules on the surface of activated platelets. This amplification system, which is characterized by an acceleration of the platelet aggregation, may be of crucial importance in haemostasis, especially as a platelet colliding with a thrombogenic surface (damaged vessel wall, activated platelet or endothelium) in flowing blood, is expected to be activated, secrete and adhere within milliseconds. Further studies will be required to look more precisely at the role of TSP in experimental thrombosis models. It is suggested that henceforth, TSP is to be considered as a potential target for developing new antithrombotic drugs, with the aim of preventing undue thrombus growth, while minimally affecting primary haemostasis.
REFERENCES


FOOTNOTES

1 The abbreviations used are: TSP, thrombospondin-1; TSP18, recombinant amino-terminal domain of TSP; Fg, fibrinogen; GPIIbIIIa*, activated $\alpha_{IIb}\beta_3$ integrin; Fg-GPIIbIIIa*-bead, Fg bound to activated $\alpha_{IIb}\beta_3$ integrin immobilized on bead; AFP, Activated Fixed Platelet; Fg-AFP, Fg bound to activated $\alpha_{IIb}\beta_3$ integrin on AFP; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; RT, room temperature; WP, washed platelets; PRP, plasma rich platelet; $K_d$, dissociation constant; $B_{max}$, maximum binding; $PA$, percentage of aggregation; $\alpha$, aggregation efficiency.
FIGURES LEGENDS

FIGURE 1

*Kinetics of FITC-TSP binding to Fg-beads*

Fg-beads (●) or BSA-beads (○) (control), (10,000/µl) were incubated with FITC-TSP (200 nM) in modified Tyrode buffer for increasing time up to 3 hours, at RT in the dark. The number of FITC-TSP molecules associated with the beads was calculated from the fluorescence bound to the beads, measured by flow cytometry. Results from one experiment representative of three separate assays.

FIGURE 2

*Inhibition of FITC-TSP binding to Fg-beads by TSP18*

Fg-beads or BSA-beads were incubated with FITC-TSP (80 nM) in presence of increasing concentrations of TSP18 (0 to 2,000 nM), for 1 h at RT in the dark. The fluorescence associated with the beads was measured by flow cytometry. The results are presented as the percentage of FITC-TSP specifically bound to Fg-beads (fluorescence measured on BSA-beads was subtracted from the fluorescence measured on the Fg-beads). Mean ± SE of three experiments.

FIGURE 3

*Binding isotherms of FITC-TSP or FITC-TSP18 to Fg-beads.* Fg-beads (●) or BSA-beads (○) were incubated with increasing concentrations of FITC-TSP (0 to 400 nM) (A) or FITC-TSP18 (0 to 2000 nM) (B) for 1 h. The fluorescence associated with the beads was measured by flow cytometry. The results are expressed as the number of FITC-TSP or FITC-TSP18 molecules bound to the beads. The binding of FITC-TSP to BSA-beads, considered as non specific, amounted to about 30% of binding to Fg-beads. The binding of FITC-TSP to the Fg-beads (mean ± SE of three experiments) is best fitted by the non-linear regression curves (1) and (2) constructed from [TSP] < 150 nM and [TSP] > 200 nM data, respectively.

FIGURE 4
Aggregation of Fg-beads by soluble TSP. Fg-beads (10,000/µl) were incubated for 20 min at RT with increasing concentrations of TSP, then sheared at 300 s⁻¹. After 120 s of shear, buffer or 4 µM of TSP18 (black or white crossed squares, respectively) was added to the Fg-beads aggregated by 200 nM TSP and samples were sheared for additional 120 s. Means ± SE of three experiments.

FIGURE 5

Binding isotherm of FITC-Fg to GPIIbIIIa*-beads. Influence of preincubation of FITC-Fg with TSP. GPIIbIIIa*-beads were incubated for 1 h in modified Tyrode buffer, with increasing concentrations of FITC-Fg (0 to 100 nM) preincubated 30 min with buffer (○) or 80 nM TSP (●). BSA-beads were used instead of GPIIbIIIa*-beads, as a control for non-specific binding. The results are expressed as the number of FITC-Fg molecules specifically bound to GPIIbIIIa*-beads. Results from one experiment representative of three separate assays. Inset: schematic representation describing the assay. : Fg; : TSP; : GPIIbIIIa*.

FIGURE 6

Binding isotherm of FITC-TSP to GPIIbIIIa*-beads or Fg-GPIIbIIIa*-beads. GPIIbIIIa*-beads (○) or Fg-GPIIbIIIa*-beads (●) were incubated with increasing concentration of FITC-TSP as described in the Experimental Procedures. Results are expressed as the number of FITC-TSP molecules bound per bead. The data from one typical experiment are shown. The curves constructed from the data correspond to the best fit equations for either a one binding site (dotted line) or a two binding site association model (solid line). Inset: schematic representation describing the assay. : Fg; : TSP; : GPIIbIIIa*.

FIGURE 7

Aggregation of Fg-GPIIbIIIa*-beads or Fg-activated fixed platelets (Fg-AFP) by soluble TSP

Fg-GPIIbIIIa*-beads (10,000/µl) (A) or Fg-AFP (40,000/µl) (B) were incubated for 30 min at RT without (○) or with (●) 180 nM or 235 nM TSP, respectively, in BAT buffer supplemented with 1 mM CaCl₂, then sheared at 300 s⁻¹ in presence of 1 µM Ro 44-9883. Results, expressed as percentage of aggregation, are the mean ± SE of three
experiments. Inset: schematic representation describing TSP-mediated aggregation of Fg-GPIIbIIIa*-beads or Fg-AFP in presence of Ro 44-9883. Fg; TSP; GPIIbIIIa*; Ro 44-9883.

FIGURE 8

Effect of the addition of TSP on aggregation efficiency of Fg-GPIIbIIIa*-beads at 0.5% receptor occupancy.

GPIIbIIIa*-beads (7000 µl) where incubated in BAT buffer, 1 mM CaCl₂, with 0.15 nM of FITC-Fg for 30 min at RT to reach 0.5% receptor occupancy. Beads were then incubated 30 min at RT with buffer (○) or 180 nM TSP (●), and sheared at 300 s⁻¹. Beads prepared with 5% Fg-receptor occupancy (▼) were sheared in parallel as a control for maximal aggregation. Mean ± SE of three experiments. Inset: schematic representation describing the assay.

Fg; TSP; GPIIbIIIa*.

FIGURE 9

Binding isotherm of FITC-TSP to TSP-beads. FITC-TSP (0 to 500 nM) was incubated with TSP-beads (●) or BSA-beads (○) (non specific binding) for 1 h at RT in the dark. Results, expressed as the number of FITC-TSP molecules bound per bead, are the mean ± SE of three experiments.

FIGURE 10

Coaggregation of TSP-beads. TSP-beads (10,000/µl), in BAT buffer supplemented with 1 mM CaCl₂, were sheared with varying the shear rate from 100 s⁻¹ to 2000 s⁻¹. TSP-beads were also sheared at 300 s⁻¹ in the presence of 8 mM EDTA (preincubated with the beads 1 min before the shear). Results, expressed as percentages of aggregated platelets (A) or aggregation efficiencies (B), are the means ± SE of at least three separate experiments.

FIGURE 11 Schematic diagram, adapted from Goldsmith et al (52), (not drawn to scale) illustrating the area available for cross-bridging with GPIIbIIIa*-Fg-GPIIbIIIa* and/or with GPIIbIIIa*-Fg-TSP-Fg-GPIIbIIIa* cross bridges on the surface of activated platelets of 1.13 µm equivalent spherical radius (53). Surface areas were calculated as published (52), using the following estimated molecular lengths: GPIIbIIIa: 10 nm (54), fibrinogen: 47
nm (55), and TSP: 54 nm (length of a single chain) (56). As shown, GPIIbIIIa*-Fg-GPIIbIIIa* cross-bridges (total length of 67.5 nm) give a maximum surface area for cross-linking of 0.20 µm² (gray area), compared to 0.56 µm² (2.8x = hatched area) in presence of GPIIbIIIa*-Fg-TSP-Fg-GPIIbIIIa* cross-bridges (total length of 169 nm).

FIGURE 12 Model for the participation of TSP in platelet aggregation mediated by Fg-GPIIbIIIa* interactions. In absence of TSP ( ), Fg ( ) directly cross-bridges GPIIbIIIa* ( ) molecules on different platelets (A); TSP could form cross-bridges between Fg bound to GPIIbIIIa*, either as a monomer (B) or multimer of n repeating units (C). TSP-(n)-TSP interactions could cross-bridging platelets via receptors ( ) other than Fg bound to GPIIbIIIa* (D). TSP could also participate in macromolecular structures generating inter- and intra-platelet cross-links favouring cluster formation on and between platelets (E).
TABLES

TABLE 1
Aggregation of AFP by Fg with varying Fg-receptor occupancy: effect of the addition of TSP on the aggregation efficiency (α) at a fixed shear rate. AFP (40,000/µl) were sheared at 300 s⁻¹ in BAT buffer supplemented with 1 mM CaCl₂, after a 30 min preincubation with Fg or a mixture of Fg and TSP, with varying the incubation time and the concentrations to reach increasing GPIIbIIIa* occupancy on AFP. Aggregation efficiencies were calculated from aggregation curves. Means ± SE of three to eleven experiments.

TABLE 2
Aggregation of AFP by Fg with varying shear rate at a fixed receptor occupancy: effect of the addition of TSP on the aggregation efficiency (α). AFP (40,000/µl) were incubated 30 min in BAT buffer supplemented with CaCl₂ (1 mM) and with Fg (20 nM), or with Fg (20 nM) together with TSP (80 nM), preincubated 15 min at room temperature to reach 20% GPIIbIIIa receptor occupancy. The platelets were then sheared in a microcouette at 300, 1000 or 2000 s⁻¹. Aggregation efficiencies were calculated from aggregation curves. Means ± SE of four to eleven experiments.

† TSP data expressed as % increase in α over that seen with Fg alone.
‡ paired t-test compared with aggregation efficiencies performed in absence of TSP.
### TABLE 1

<table>
<thead>
<tr>
<th>Receptor occupancy (%)</th>
<th>$\alpha$ with Fg only (%)</th>
<th>Effect of addition of TSP on $\alpha$ (% increase) $^\dagger$</th>
<th>Paired t-test $^\ddagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-6</td>
<td>8.6 ± 0.9</td>
<td>+ 41.2 ± 11.7</td>
<td>p&lt;0.01 (n=8)</td>
</tr>
<tr>
<td>20</td>
<td>14.5 ± 1.7</td>
<td>+ 30.0 ± 5.5</td>
<td>p&lt;0.003 (n=11)</td>
</tr>
<tr>
<td>35</td>
<td>20.1 ± 4.4</td>
<td>+ 61.3 ± 38.0</td>
<td>p&lt;0.07 (n=3)</td>
</tr>
</tbody>
</table>

### TABLE 2

<table>
<thead>
<tr>
<th>Shear rate (s$^{-1}$)</th>
<th>$\alpha$ with Fg only (%)</th>
<th>Effect of addition of TSP on $\alpha$ (% increase) $^\dagger$</th>
<th>Paired t-test $^\ddagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>14.5 ± 1.7</td>
<td>+ 30.0 ± 5.5</td>
<td>p&lt;0.003 (n=11)</td>
</tr>
<tr>
<td>1000</td>
<td>4.7 ± 1.4</td>
<td>+ 28.8 ± 23.2</td>
<td>p&lt;0.3 (n=4)</td>
</tr>
<tr>
<td>2000</td>
<td>1.0 ± 0.2</td>
<td>+ 110.6 ± 60.0</td>
<td>p&lt;0.1 (n=4)</td>
</tr>
</tbody>
</table>
FIGURE 1

![Graph showing FITC-TSP molecules/ bead over time (min).](image-url)
FIGURE 4

[Graph showing aggregation percentage over time with different concentrations of TSP18]
FIGURE 9

[Graph showing the relationship between FITC-TSP molecules/bead and FITC-TSP (nM).]
FIGURE 12
A model of platelet aggregation involving multiple interactions of Thrombospondin-1, Fibrinogen and GPIIbIIIa receptor
Arnaud Bonnefoy, Roy Hantgan, Chantal Legrand and Mony M. Frojmovic

J. Biol. Chem. published online November 27, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M010091200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2000/11/27/jbc.M010091200.citation.full.html#ref-list-1