

# Thrombospondin Binds to Monocytes–Macrophages and Mediates Platelet–Monocyte Adhesion

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## Abstract

Thrombospondin (TSP) is a multifunctional platelet glycoprotein synthesized by a variety of cells in culture including monocytes and macrophages. We now report that  $^{125}\text{I}$ -TSP binds specifically, saturably, and reversibly to mouse peritoneal macrophages and to cells of the monocyte-like human cell line U937 with dissociation constants of  $6.7\text{--}14.5 \times 10^{-8} \text{ M}$  and  $3\text{--}4 \times 10^5$  binding sites per cell. TSP mediates an adhesive interaction between thrombin-stimulated platelets and both U937 cells and human blood monocytes. Using a sensitive rosetting assay, we found that monocytes were not rosetted by resting platelets whereas  $> 90\%$  were rosetted by thrombin-stimulated platelets. Monoclonal and polyclonal anti-TSP antibodies markedly inhibited rosetting as did TSP itself. Neither control antibodies nor heparin, fibronectin, fibrinogen, nor the fibronectin adhesion tetrapeptide Arg-Gly-Asp-Ser inhibited rosetting. TSP may thus serve as a molecular bridge linking activated platelets with monocytes at sites of early vascular injury. Such interaction may be of critical importance in the regulation of thrombosis and the initiation of atherosclerosis.

## Introduction

Thrombospondin (TSP)<sup>1</sup> is a 450-kD trimeric glycoprotein with a broad biological distribution (1). Although circulating plasma concentrations are low (2), it is a major  $\alpha$ -granule protein of human platelets (3) and upon stimulation is expressed on the platelet surface where it stabilizes platelet aggregation (4). In addition, TSP is synthesized by fibroblasts, endothelial cells, aortic smooth muscle cells, and glial cells in culture and is incorporated into their extracellular matrices (5–8). The biological role of extra-platelet TSP is unknown, although it has been shown to interact specifically with several macromolecules including fibrinogen, fibronectin (FN), type V collagen, and heparin (9–12) and to have lectin-like activity (13). We have found that

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1. *Abbreviations used in this paper:* FN, fibronectin;  $K_d$ , dissociation constant; mAb, monoclonal antibody; PBM, peripheral blood monocyte; RGDS, fibronectin adhesion tetrapeptide Arg-Gly-Asp-Ser; TSP, thrombospondin; vWF, von Willebrand factor.

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TSP binds specifically to plasminogen (14) and histidine-rich glycoprotein (15) and can assemble multimolecular complexes involving plasminogen, histidine-rich glycoprotein, heparin, and tissue plasminogen activator (16, 17). These complexes can modulate the function of the bound ligands, for example, by inhibiting the anticoagulant effect of heparin and allowing efficient plasminogen activation in the absence of fibrin. Such extravascular plasmin generation may have importance in effecting such physiological and pathological events as tumor cell migration and implantation, inflammation, and embryogenesis (18). Recently monocytes and tissue macrophages have been found to produce and secrete TSP (19). Experimental evidence strongly suggests that blood monocytes may play an important role in initiating thrombosis and atherosclerosis. For example, among the earliest changes in experimental atherosclerosis are monocyte adhesion to endothelial cells, transendothelial passage, and ultimate lipid accumulation to form foam cells (20, 21). In addition, monocytes have been shown to express tissue factor and to participate effectively in fibrin clot formation (22). It is possible that TSP, a protein with known lectin-like properties (13), may play a role in mediating interactions of monocytes with other vascular cells, and by virtue of its ability to assemble biologically active multiprotein complexes, may play a role in effecting these processes. To define the role of TSP in monocyte biology, we have studied U937 cells, a line derived originally from a patient with histiocytic lymphoma, which express phenotypic features most consistent with monocyte lineage (23) and are used by others as a model of monocyte biology (24, 25). We find that TSP binds specifically to the surface of U937 cells and murine peritoneal macrophages and mediates the adhesive interaction of thrombin-stimulated human platelets with these cells.

## Methods

### Materials

Porcine intestinal heparin sodium was obtained from Elkins-Sinn, Inc., Cherry Hill, NJ; Ficoll-Hypaque and density Percoll gradients from Pharmacia Fine Chemicals, Piscataway, NJ; carrier-free  $^{125}\text{I}$ -NaI from New England Nuclear, Boston, MA; the aminosugars  $\alpha$ -D-glucosamine,  $\alpha$ -D-mannosamine,  $\alpha$ -D-acetylglucosamine, and  $\alpha$ -D-acetyl mannosamine from Calbiochem-Behring Corp., La Jolla, CA; thioglycollate broth from Difco Laboratories, Inc., Detroit, MI; and phorbol 12-myristate 13-acetate (PMA) from Sigma Chemical Co., St. Louis, MO. Sterile tissue culture plasticware was purchased from Falcon Labware, Becton, Dickinson & Co., Cockeysville, MD. All reagents were of analytic grade.

### Purified proteins

Purified human calcium-replete TSP was prepared from ionophore A23187-induced platelet releasate as previously described (16) by sequential heparin-Sepharose affinity chromatography and anion-exchange chromatography using a Mono Q column and a fast-pressure liquid chromatography system (Pharmacia Fine Chemicals). Human plasminogen-free fibrinogen (Imco, Stockholm) was purchased from MICO, USA, New York; bovine serum albumin (Pentex BSA) from Miles Laboratories,

Naperville, IL, and human albumin from Calbiochem-Behring Corp. Human FN was gift of Dr. D. Falcone (Cornell University Medical College), and human thrombin was a gift of Dr. J. Fenton II, New York State Department of Health, Albany, NY. The tetrapeptide Arg-Asp-Ser-Gly (RGDS) was obtained from Peninsula Laboratories, Belmont, CA. Purified TSP, human albumin, rabbit anti-TSP Fab, and mouse monoclonal anti-TSP IgG were labeled with  $^{125}\text{I}$  by the modified chloramine-T method as previously described (14).

### Antibodies

Anti-TSP and anti-FN antisera were raised in rabbits and IgG was isolated from the sera by DEAE-agarose-Cebacron Blue (Pharmacia Fine Chemicals) chromatography as previously described (9). The anti-TSP IgG was affinity-purified using insolubilized TSP and Fab fragments generated by papain digestion according to the method of Porter (26). These did not react with fibrinogen, FN, or von Willibrand factor (vWF). Normal rabbit Fab, normal mouse IgG, and fluorescein-conjugated goat anti-rabbit and anti-mouse IgG were purchased from Cappel Laboratories, Cochranville, PA. Murine monoclonal anti-TSP antibodies (mAbs) were made by standard techniques modified from Kohler and Milstein (27). Mice were immunized with purified TSP in alum by multiple intraperitoneal injections. Splenocytes were then fused with cells of the nonsecretory murine myeloma cell line SP 2/0 and positive hybridomas selected by enzyme-linked immunosorbent assay (ELISA) using TSP immobilized on polystyrene microtiter wells. Positive hybridomas were subcloned into soft agar, rescreened, and then propagated in mouse ascites culture. The mAb IgGs were then obtained by precipitation with 50% saturated ammonium sulfate. Three monoclonal anti-TSP IgGs, designated 11.4, 45.2, and 46.4, were used in these studies. These did not react by ELISA with FN, fibrinogen, or vWF. All three bound to thrombin-stimulated platelets using indirect immunofluorescent techniques and a fluorescence-activated cell sorter (data not shown). An anti-lymphocyte antibody was used as a control. None of these three monoclonal antibodies reacts with TSP or proteolytic fragments of TSP by Western blot, suggesting that they recognize an epitope(s) determined by the native tertiary structure of the molecule. To characterize these antibodies further, their effect on platelet aggregation was also studied. At concentrations from 10 to 500  $\mu\text{g}/\text{ml}$ , none of them inhibited aggregation induced by thrombin (0.1–0.5 U/ml). Aggregation studies were performed as described by Dixit et al. (29) with a dual-channel aggregometer (Payton Scientific Inc., Buffalo, NY) using fresh gel-filtered human platelets. In addition, we used murine monoclonal anti-TSP IgG C6.7, a generous gift of Dr. Vishva Dixit (University of Michigan, Ann Arbor). This antibody has been shown to react with an epitope near the carboxy terminus of the TSP molecule (28) and to inhibit thrombin-induced platelet aggregation (29).

### Cells and cell culture

U937 cells were obtained from Dr. J. Unkeless (Mt. Sinai Medical College, New York) and were maintained in culture in RPMI 1640 medium (M.A. Bioproducts, Walkersville, MD) supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50  $\mu\text{g}/\text{ml}$  streptomycin. Cells were subcultured 1:4 three times per week into 75-cm<sup>2</sup> flasks. In some studies U937 cells were differentiated to a more macrophage-like phenotype by incubating  $0.5 \times 10^6$  cells/ml in medium containing PMA  $10^{-8}$  M for 48 h (30).

Resident and elicited murine macrophages were obtained from the peritoneal cavities of Nelson-Collins strain female mice (Rockefeller University) as described by Edelson and Cohn (31). Activated cells were elicited by intraperitoneal injection of 1 ml of 3% thioglycollate broth in 20 mM phosphate-buffered saline. 4 d after injection, elicited macrophages and a resident cell population from uninjected mice were harvested in Dulbecco's modified Eagle's medium (DME) containing penicillin and streptomycin, washed, resuspended in DME containing 20% fetal calf serum, and dispensed into 24-well sterile plates at  $0.5 \times 10^6$  cells per well. Wells were then incubated for 2 h and the nonadherent cells were removed by washing three times in DME plus fetal calf serum. Cells were maintained in culture for 24–48 h before use.

Human peripheral blood monocytes (PBMs) were isolated according

to the method of Wright et al. (32). Leukocyte-rich fractions purchased from the Greater New York Blood Center were washed twice in divalent cation-free Hanks' balanced salt solution (HBSS), applied to Ficoll-Hypaque, and centrifuged at 400 g for 40 min. The mononuclear cell-rich fraction was then washed three more times in HBSS at 200 g for 10 min to decrease platelet contamination. The cells were then applied to a density Percoll gradient and the monocyte-containing fraction was washed, resuspended in RPMI 1640 containing penicillin, streptomycin, and 10% type-specific human serum, and plated into 100-cm<sup>2</sup> dishes. After 1 h the nonadherent cells were removed, and the flasks were washed and then reincubated in RPMI 1640/10% human serum for 1–7 d before use. For platelet-rosetting studies the adherent monocytes were removed from washed flasks by incubating in ice-cold HBSS for 1 h at 4°C followed by gentle scraping with a rubber policeman. The cells were then counted, pelleted by centrifugation, and resuspended in RPMI 1640 containing 2 mg/ml BSA. Lymphocytes were obtained from anticoagulated whole blood treated as above (save the Percoll gradient) and taking the nonadherent cells. These were washed three times in RPMI 1640 and used immediately.

Human platelets were isolated from fresh citrated whole blood by centrifugation at 200 g for 10 min followed by gel filtration on a column of Sepharose 2B (Pharmacia Fine Chemicals) in a divalent cation-free modified Tyrode-Hepes buffer (0.14 M NaCl, 2.7 mM KCl, 0.42 mM  $\text{NaH}_2\text{PO}_4$ , 12 mM  $\text{NaHCO}_3$ , 5.5 mM glucose, 5 mM Hepes, pH 7.4) (33). Platelets were pooled, stimulated under nonstirring conditions with human thrombin (0.1 U/ml) for 30 min at 22°C and then lightly fixed with 1.3% formaldehyde at 4°C for 60 min according to the method of Jungi et al. (34). The fixed platelets were then washed three times and resuspended in buffer containing 2 mg/ml BSA and were used within 72 h of preparation. In some studies fresh gel-filtered human platelets were used in place of fixed platelets.

### $^{125}\text{I}$ -TSP binding assays

**U937 cells.** U937 cells were washed three times, resuspended at  $2 \times 10^6$  cells/ml, and then incubated in serum-free medium made up of RPMI 1640 containing 2 mg/ml BSA (RPMI/BSA) before use. The cells were then rewashed and aliquoted into 1.5-ml polypropylene microcentrifuge tubes ( $0.5\text{--}1.0 \times 10^6$  cells per aliquot; 0.1–0.15 ml per tube). To these were added radioiodinated ligands (final volume 0.15–0.25 ml) diluted also in RPMI/BSA. After incubation, the cell suspensions were layered onto 0.4 ml of silicone oil (67:33 vol/vol of Dow Corning 550 and 556 oils, Dow Corning Corp., Midland, MI) and centrifuged at 14,000 g in a tabletop microfuge (Beckman Instruments, Inc., Fullerton, CA). The aqueous phase was removed and nonbound radioactivity was counted. The tubes were then inverted and the tip containing the cell pellet was amputated and placed in a gamma counter to assess cell-bound radioactivity. To demonstrate reversibility, cells were incubated with  $^{125}\text{I}$ -TSP for 2 h at 4°C, then washed, and resuspended in RPMI/BSA containing unlabeled TSP (200  $\mu\text{g}/\text{ml}$ ). At timed intervals bound and free radioactivity was measured as above. For all binding assays, cell viability was assessed by dye exclusion and was > 95%.

Binding of  $^{125}\text{I}$ -conjugated rabbit anti-TSP Fab and murine anti-TSP monoclonal IgG 11.4 to U937 cells was also measured as described above. In these studies nonspecific binding was determined as the amount of bound radioactivity in the presence of a 100-fold excess of unlabeled ligand. Antibody binding was also measured using an indirect immunofluorescence assay. In these studies anti-TSP antibodies were added to the cells which were then washed, fixed with formaldehyde, and then incubated with fluorescein-conjugated second antibodies. After washing, bound antibody was detected by microscopy or by fluorescence activated cell sorter. Thrombin-stimulated platelets served as positive controls.

**Macrophages.** Tissue culture wells containing adherent macrophages were washed three times and incubated for 2 h in DME containing 2 mg/ml BSA. The wells were then rewashed and to them were added radioiodinated ligands in 0.2 ml DME/BSA. After incubation the media was removed and nonbound radioactivity was counted. After extensive washing, the bound material was removed and counted by solubilizing well contents in 0.2 N NaOH. This procedure removed > 99% of the

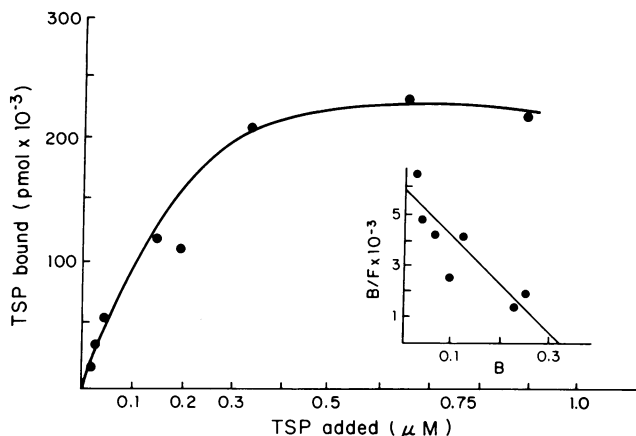
cells and radioactivity. In some wells cell counts were determined in parallel by the technique of Bohnsack et al. (35). Plasma membranes of the adherent cells were lysed with a stromatolysing agent (Zap-oglobin II, Coulter Diagnostics, Hialeah, FL) and the released nuclei were counted with a Coulter counter (Coulter Electronics, Hialeah, FL). To assess reversibility, wells containing adherent macrophages were incubated with  $^{125}\text{I}$ -TSP for 2 h at 4°C. Wells were then washed and fresh DME/BSA containing unlabeled TSP added (200  $\mu\text{g}/\text{ml}$ ). At timed intervals bound and free radioactivity was assessed as above.  $^{125}\text{I}$ -conjugated anti-TSP antibody binding and anti-TSP immunofluorescence microscopy was also performed with these cells.

### Platelet-monocyte adhesion assay

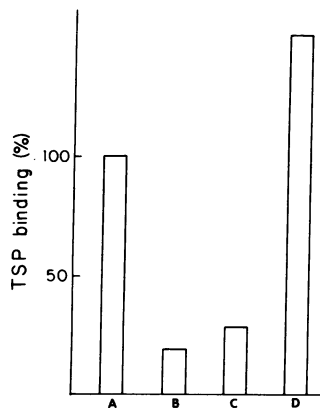
Platelet-monocyte interactions were quantified with a rosetting assay modified after the method of Jungi et al. (34). 50  $\mu\text{l}$  of gel-filtered, thrombin-stimulated, fixed platelets ( $3\text{--}5 \times 10^7$  platelets) were incubated with 100  $\mu\text{l}$  of U937 cells ( $3\text{--}5 \times 10^5$  cells) or peripheral blood monocytes ( $3\text{--}5 \times 10^5$  cells) rotating end-over-end in a  $12 \times 75\text{-mm}$  polypropylene tube at 4°C. The cells were then placed in a Neubaur chamber and examined by phase-contrast light microscopy. Platelet-monocyte adhesion was quantified by counting at least 200 mononuclear cells and scoring for the presence of platelet rosetting (more than two adherent platelets per mononuclear cell). In addition, the number of platelets adherent to 20–40 rosetted cells was counted. In some experiments, before mixing with the mononuclear cells, the platelets were preincubated for 30 min with polyclonal anti-TSP Fab (100  $\mu\text{g}/\text{ml}$ ), monoclonal anti-TSP IgG (10  $\mu\text{g}/\text{ml}$ ), or control antibodies, with the aminosugars  $\alpha\text{-D}$ -mannosamine and galactosamine and their acetylated derivatives (57 mM), with sodium heparin (20  $\mu\text{g}/\text{ml}$ ) or with purified TSP (0.33  $\mu\text{M}$ ). In others, the mononuclear cells were preincubated with purified TSP (0.33  $\mu\text{M}$ ), control proteins, RGDS peptide (0.12 mM), the aminosugars, or the antibodies. As controls, resting platelets were substituted for stimulated platelets and lymphocytes were substituted for monocytes. Geimsa stains were done to confirm morphologic identity of the mononuclear cells. Some studies were done with live unfixed platelets. These were done as described above except the mononuclear cell-platelet incubation was done at 22°C under nonstirring conditions.

## Results

**TSP binds specifically to U937 cells.**  $^{125}\text{I}$ -TSP bound to U937 cells in a specific and concentration-dependent manner, as measured using a suspension binding assay (Fig. 1). At 4°C, binding



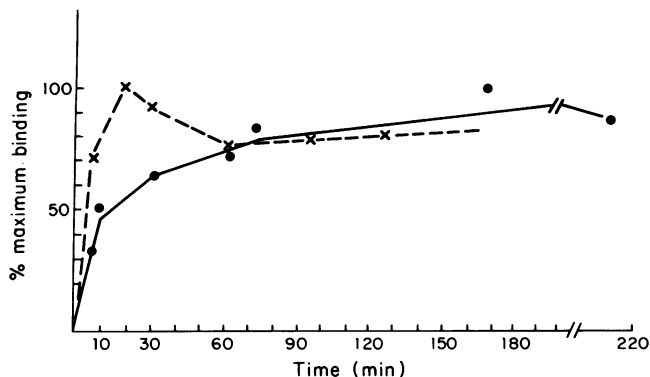
**Figure 1.** Binding of  $^{125}\text{I}$ -TSP to U937 cells. U937 cells ( $0.5 \times 10^6$ ) were incubated with  $^{125}\text{I}$ -TSP ( $1.9 \times 10^4$  cpm/ $\mu\text{g}$ ) in RPMI/BSA for 2 h rotating at 4°C. Bound and free ligands were then separated by centrifugation (14,000 g for 5 min) through silicone oil and radioactivity was counted. Each point represents the mean of duplicate tubes. (*Inset*) Scatchard plot calculated using a least squares linear regression.



**Figure 2.** Effect of divalent cations, heparin sulfate, and excess unlabeled ligand on  $^{125}\text{I}$ -TSP binding to U937 cells.  $^{125}\text{I}$ -TSP (20  $\mu\text{g}/\text{ml}$ ) was incubated with U937 cells for 2 h rotating at 4°C in RPMI/BSA containing (A) 1 mM additional  $\text{CaCl}_2$  and  $\text{MgCl}_2$ , (B) 5 mM EDTA, (C) 400  $\mu\text{g}/\text{ml}$  unlabeled TSP, or (D) 20  $\mu\text{g}/\text{ml}$  heparin sulfate. Binding was measured after centrifugation through silicone oil and is expressed as percentage of that in panel A.

saturated at  $\sim 0.3 \mu\text{M}$  and Scatchard analysis (Fig. 1, *inset*) revealed a dissociation constant ( $K_d$ ) of 145 nM with  $\sim 3 \times 10^5$  binding sites per cell. Computer-assisted data analysis using the ligand program of Munson and Rodbard (36) for nonlinear curve fitting gave the best fit for a single class of binding sites. At an input concentration of 0.04  $\mu\text{M}$   $^{125}\text{I}$ -TSP, co-incubation with a 20-fold excess of unlabeled ligand inhibited 75% of radioactive protein binding (Fig. 2), demonstrating specificity. In addition,  $^{125}\text{I}$ -human albumin did not bind to these cells, nor did  $^{125}\text{I}$ -TSP bind to control cells (human lymphocytes), further demonstrating specificity. Binding of TSP to U937 cells was optimal in the presence of calcium and magnesium and was 82% inhibited by 5 mM EDTA (Fig. 2). Heparin sulfate, which inhibits binding to TSP to sulfated glycolipids (37), did not inhibit binding of TSP to U937 cells; in fact, binding was augmented  $\sim 50\%$  by 20  $\mu\text{g}/\text{ml}$  heparin (Fig. 2). U937 cells stimulated with PMA ( $10^{-8}$  M for 48 h) bound TSP to an equivalent extent and affinity as unstimulated cells (data not shown). These cells demonstrated morphologic criteria of stimulation, i.e., adherence and pseudopod formation.

TSP binding to U937 cells at 4°C was time dependent (Fig. 3), reaching a steady state by 30–45 min. At 37°C, binding was more rapid, but at steady state the extent of binding at the two temperatures was equivalent. 80% of the surface-bound  $^{125}\text{I}$ -TSP was driven off in a time-dependent manner by resuspending the



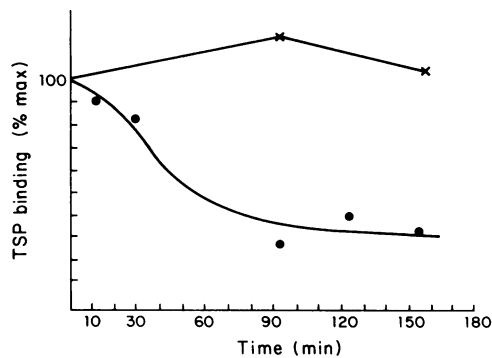
**Figure 3.** Time dependence of TSP binding to U937 cells. U937 cells were incubated at 4°C (solid circles) or 37°C (x) with  $^{125}\text{I}$ -TSP (50  $\mu\text{g}/\text{ml}$ ) for timed intervals as in Fig. 1. Bound radioactivity was then determined after centrifugation through silicone oil and is expressed as percentage of maximum. Points are means of duplicate tubes.

cells in unlabeled TSP (Fig. 4), demonstrating reversibility. This reversibility occurred even after initial incubations of up to 3 h.

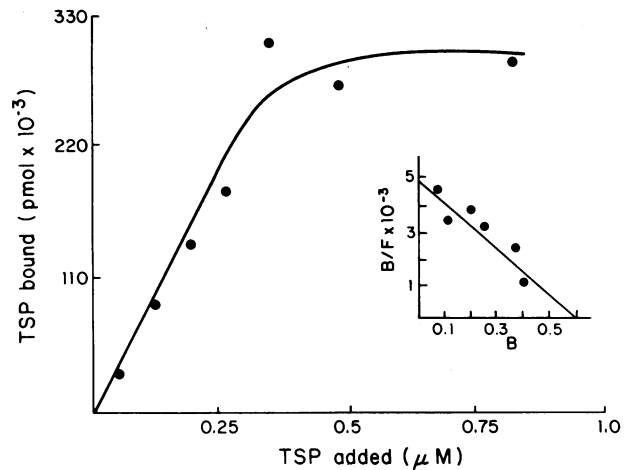
*TSP binds specifically to murine peritoneal macrophages.* Using a solid-phase binding assay, we showed that  $^{125}\text{I}$ -TSP binds specifically to murine peritoneal macrophages adherent to tissue culture wells. Binding was concentration dependent (Fig. 5), reaching saturation at  $\sim 0.3 \mu\text{M}$  TSP. Scatchard analysis (Fig. 5, inset), calculated as described for the U937 cells, revealed a  $K_d$  of 67 nM with  $\sim 4 \times 10^5$  binding sites per cell. As with U937 cells, analysis by the ligand program gave best fit for a single class of binding sites. For the purposes of these studies, it was assumed that the entire cell surface was available to the TSP. The number of binding sites may therefore be underestimated.  $^{125}\text{I}$ -human albumin did not bind to these cells, nor did  $^{125}\text{I}$ -TSP bind to cell-free wells incubated in parallel with culture medium or to wells incubated with nonadherent peritoneal cells, demonstrating specificity. Macrophages activated by prior intraperitoneal injections of thioglycollate bound TSP to an equivalent extent and affinity as nonstimulated cells (data not shown). The stimulated cells fulfilled morphologic and numerical criteria of activation.

As with the U937 cells, binding of TSP to the macrophages was time dependent and divalent cation dependent, and at low input concentrations was partially inhibited by 10-fold excess unlabeled TSP. Binding of TSP to these cells was also reversible in a time-dependent manner (Fig. 6). By 2 h, 75% of the cell-bound  $^{125}\text{I}$ -TSP was driven off after the wells were washed and reincubated with unlabeled TSP.

Because monocytes/macrophages produce TSP (19), it is possible that endogenous TSP could bind to the surface of these cells and thereby mask the presence of a low number of high affinity binding sites. Binding of anti-TSP antibodies to both U937 cells and murine peritoneal macrophages was therefore studied. Neither  $^{125}\text{I}$ -conjugated rabbit anti-TSP Fab nor murine monoclonal 11.4 bound specifically to U937 cells or murine peritoneal macrophages. In addition, indirect immunofluorescence studies using the same antibodies failed to demonstrate TSP on the surface of these cells. Positive controls were provided by thrombin-stimulated platelets. Thus no endogenous TSP under these conditions could be detected on these cells.

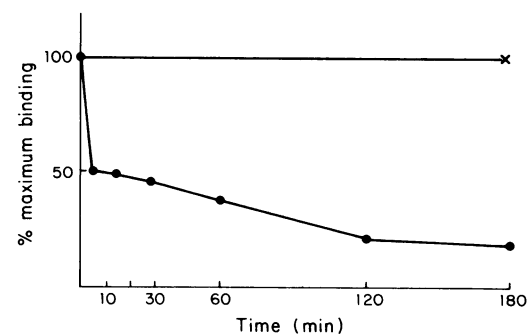


**Figure 4.** Reversibility of TSP binding to U937 cells. U937 cells were incubated with  $^{125}\text{I}$ -TSP ( $50 \mu\text{g}/\text{ml}$ ) for 2 h at  $4^\circ\text{C}$  as in Fig. 1. The cells were then pelleted by centrifugation and resuspended in RPMI/BSA ( $\times$ ) or RPMI/BSA containing unlabeled TSP ( $200 \mu\text{g}/\text{ml}$ ) (solid circles). Bound and free radioactivity was then measured after centrifugation through silicone oil. Data is plotted as percentage of bound radioactivity released. Points are means of duplicate tubes.

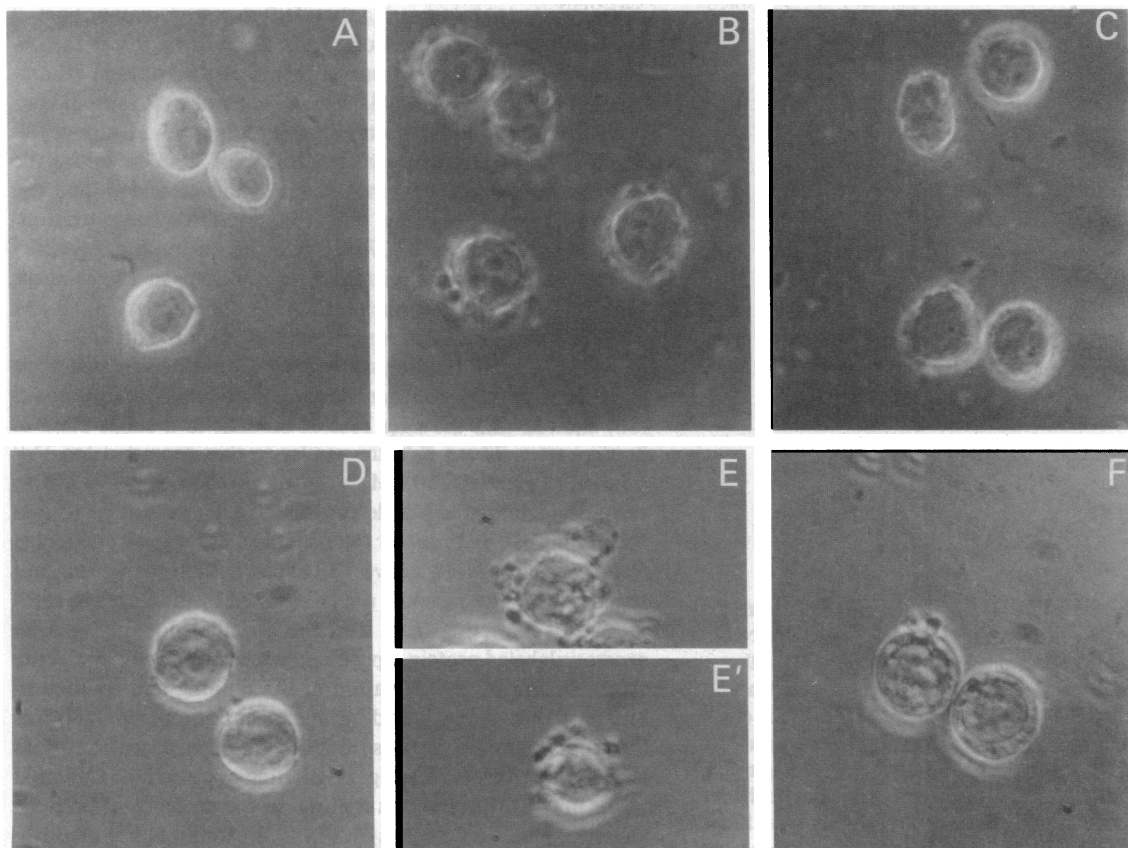


**Figure 5.** Binding of  $^{125}\text{I}$ -TSP to murine resident peritoneal macrophages. Adherent murine peritoneal macrophages maintained in culture for 24 h were incubated with  $^{125}\text{I}$ -TSP ( $2.6 \times 10^4 \text{ cpm}/\mu\text{g}$ ) in DME/BSA for 2 h rocking at  $4^\circ\text{C}$ . Wells were then washed three times and bound radioactivity was counted after solubilizing well contents in 0.2 N NaOH. Each point represents mean of duplicate wells. (Inset) Scatchard plot calculated using least squares linear regression.

*TSP mediates the adhesive interaction between thrombin-stimulated platelets and monocytes.* As recently described by Jungi et al. (34) fixed, thrombin-stimulated platelets specifically rosette human PBM and U937 cells. Platelets rosetted  $> 90\%$  of U937 cells (Fig. 7 B) and PBM but did not rosette human lymphocytes, demonstrating specificity. Resting platelets (Fig. 7 A) rosetted  $< 2\%$  of U937 cells and  $< 15\%$  of PBM. In addition, platelets stimulated with low concentrations of ADP ( $20 \mu\text{M}$ ) did not rosette, suggesting that the interaction requires platelet secretion. As shown in Fig. 7 C and Fig. 8, when fixed thrombin-stimulated platelets were preincubated with anti-TSP Fab, rosette formation was markedly inhibited (decreased to  $35 \pm 9.8\%$ ). In addition, the mean number of platelets associated with each positive cell was decreased from  $10 \pm 2.3$  platelets per cell to  $3.9 \pm 2.4$  platelets per cell. Nonimmune or anti-FN Fab did not



**Figure 6.** Reversibility of TSP binding to murine macrophages. Adherent murine peritoneal macrophages were incubated with  $^{125}\text{I}$ -TSP ( $50 \mu\text{g}/\text{ml}$ ) for 2 h at  $4^\circ\text{C}$ . Wells were then washed and fresh medium containing buffer ( $\times$ ) or unlabeled TSP ( $200 \mu\text{g}/\text{ml}$ ) was added. At timed points released radioactivity was counted. Data is expressed as percentage of total bound radioactivity (measured as in Fig. 5) released into the media. Points are means of duplicate wells.



**Figure 7.** Effect of anti-TSP Fab on platelet-U937 rosette formation. U937 cells were incubated for 30 min with (A) fixed resting platelets, (B) fixed thrombin-stimulated platelets, (C) fixed thrombin-stimulated platelets preincubated with anti-TSP Fab (100  $\mu\text{g}/\text{ml}$ ), (D) live resting platelets, (E and E') live thrombin-stimulated platelets, and (F) live

thrombin-stimulated platelets preincubated with anti-TSP Fab (100  $\mu\text{g}/\text{ml}$ ). For studies with fixed platelets, incubations were done rotating at 4°C whereas with live platelets incubations were done at 22°C under nonstirring conditions. Fresh wet mounts were examined by phase-contrast microscopy ( $\times 1,000$ ).

inhibit rosetting ( $91\pm 3\%$  and  $87\pm 3\%$ , respectively) demonstrating specificity. In addition, a panel of three murine monoclonal anti-TSP IgGs (11.4, 45.2, and 46.4) also inhibited rosette formation ( $32\pm 2\%$ ) whereas normal mouse IgG did not ( $86\pm 4\%$ ). Monoclonal anti-TSP antibody C6.7 at concentrations up to 500  $\mu\text{g}/\text{ml}$  did not inhibit rosette formation ( $90.4\pm 1.8\%$  positive). Similarly, when U937 cells were preincubated with ligand (TSP 0.33  $\mu\text{M}$ ), the number of rosetted cells was decreased to  $13\pm 7\%$ , and the mean number of platelets adherent to positive cells was decreased to  $4.3\pm 2.0$  platelets per cell (Fig. 8). Fibrinogen, FN, and the FN adhesion tetrapeptide RGDS (38) did not affect rosetting. Aminosugars, which inhibit the platelet lectin activity of TSP (13), did not inhibit rosette formation ( $92\pm 3\%$ ), nor did heparin sulfate (93%). When opposite cell types were preincubated with antibody or TSP, washed, and then studied, no inhibition of rosette formation was seen; i.e., 92% of U937 cells preincubated with anti-TSP, washed, and mixed with thrombin-stimulated platelets were rosetted. Similarly, 87% of U937 cells were rosetted by platelets preincubated with TSP and washed before mixing. These data, along with the antibody binding data, are most consistent with the hypothesis that platelet surface-associated TSP interacts with a previously unoccupied binding site on the monocyte surface, effecting an adhesive interaction.

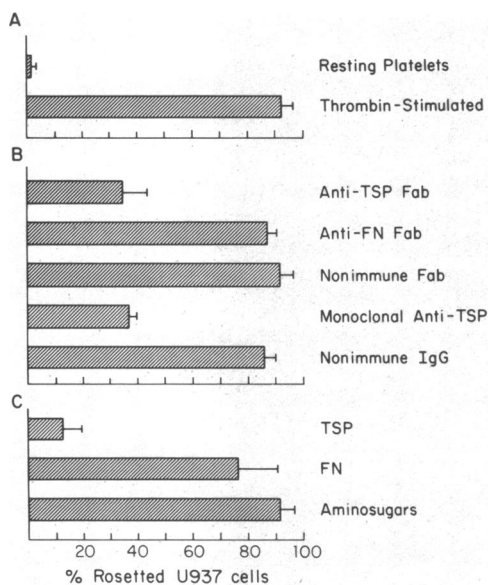
Because fixed platelets may not resemble live platelets in all aspects, some of the rosetting studies were done substituting fresh live gel-filtered platelets for fixed. As shown in Fig. 7 D,

live resting platelets did not rosette U937 cells, whereas live thrombin-stimulated platelets did (Fig. 7 E and E'). As with fixed platelets, anti-TSP Fab (Fig. 7 F), but not control Fabs markedly inhibited the interaction.

Fig. 9 shows data obtained with human PBM in the platelet rosetting assay and shows specific inhibition by anti-TSP and purified TSP comparable to that with the U937 cells. When thrombin-stimulated platelets were preincubated with anti-TSP Fab before incubation with PBM, the number of rosetted cells decreased from  $92\pm 1\%$  to  $22\pm 2\%$ . In addition, the mean number of platelets associated with each rosetted PBM decreased from  $8.9\pm 3.4$  to  $4.5\pm 3.1$  per cell. Anti-Fn Fab did not inhibit. Similarly, when PBM were preincubated with purified TSP (0.33  $\mu\text{M}$ ) before adding platelets, the number of rosetted cells decreased to  $15\pm 3\%$  compared with  $82\pm 6\%$  for FN.

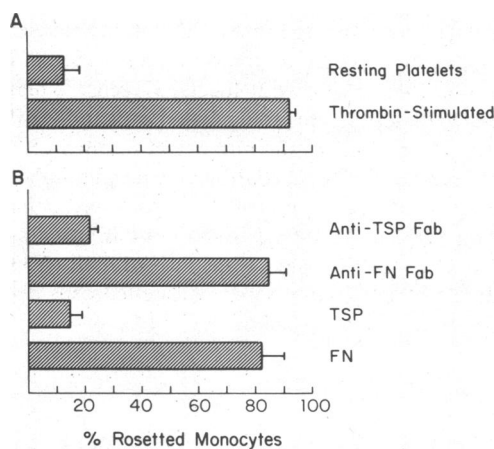
## Discussion

Although TSP is a major platelet  $\alpha$ -granule protein (3), is produced by a variety of cells in culture (5-8), and is a component of the extracellular matrix (39), the biological role of this multifunctional glycoprotein is largely unknown. An adhesive function is suggested by its lectin activity (13) (it agglutinates fixed erythrocytes) and its demonstrated role in stabilizing platelet aggregation (4). In addition, Roberts et al. (40) have recently



**Figure 8.** Role of TSP in mediating platelet-U937 cell interactions. U937 cells and platelets were incubated as in Fig. 7, placed in a Neubaur chamber and examined under phase contrast. 200 U937 cells were scored for presence of platelet rosetting (defined as more than two associated platelets per cell). Data is expressed as mean  $\pm$  SD of at least three separate experiments. (A) Interaction of gel-filtered platelets with U937 cells. (B) Effects of polyclonal anti-TSP Fab (100  $\mu$ g/ml), monoclonal anti-TSP IgG (10  $\mu$ g/ml), or control antibodies on the interaction of thrombin-stimulated platelets with U937 cells. (C) Effects of purified TSP (0.33  $\mu$ M), FN (0.33  $\mu$ M) or aminosugars (57 mM) on the interaction of thrombin-stimulated platelets with U937 cells.

reported that TSP may mediate the adhesion of falciparum malaria-infected erythrocytes to melanoma cells. It is of interest that unlike FN, endothelial cell and smooth muscle cell TSP production and matrix incorporation are increased at low cell density (and in the latter case by platelet-derived growth factor



**Figure 9.** Role of TSP in mediating platelet-PBM interactions. PBM and platelets were incubated as in Fig. 8. Data is expressed as mean percentage of rosetted cells ( $\pm$ SD). (A) Interaction of gel-filtered platelets with PBM. (B) Effects of anti-TSP Fab (100  $\mu$ g/ml) and purified TSP (0.33  $\mu$ M) on the interaction of thrombin-stimulated platelets with PBM.

stimulation) (41), suggesting that TSP secretion may be part of the injury response of vascular cells. Supporting this is the observation that TSP is present in increased amounts in atherosclerotic vessel walls (42, 43). We now demonstrate specific saturable binding of TSP to tissue macrophages and monocyte-like U937 cells and demonstrate a role for TSP in mediating the adhesive interaction between stimulated platelets and monocytes.

Figs. 1 and 5 show that  $^{125}$ I-TSP bound in a concentration-dependent manner to U937 cells in a suspension binding assay and to murine resident peritoneal macrophages in a solid-phase binding assay. In both cases saturation occurred at  $\sim$  0.3  $\mu$ M TSP. Scatchard plots (*insets*) revealed  $K_d$ 's on the order of  $10^{-7}$  M with  $3-4 \times 10^5$  binding site per cell. Computer-assisted Scatchard analysis using the ligand program of Munson and Rodbard (36) showed that the data best fit binding to a single class of binding sites. These  $K_d$ 's are similar to that reported for TSP binding to platelets (50 nM to resting platelets and 250 nM to thrombin-stimulated platelets) (44) and are also similar to that for binding of TSP to tumor cell lines (45). Because we are unable to prepare biologically active TSP at concentrations  $>$  1 mg/ml, specificity of binding could not be assessed by the standard technique of subtracting from total binding that not inhibited by excess unlabeled protein. Specificity was shown, however, by the saturable and reversible nature of the binding and by the observation that, at low ligand input concentrations, up to 80% of the bound radioactivity was inhibited in the presence of 20-fold excess of unlabeled TSP (Fig. 2). Binding was also 80% inhibited by 10 mM EDTA and was optimal in the presence of both divalent cations,  $Ca^{2+}$  and  $Mg^{2+}$  (Fig. 2). In addition,  $^{125}$ I-albumin did not bind to the cells, nor did  $^{125}$ I-TSP bind to lymphocytes.

Binding of TSP to monocytes and macrophages was time dependent and reached equilibrium by 45 min at both 37°C and 4°C (Fig. 3). There was no difference in extent of binding at either temperature. As shown in Figs. 4 and 6, up to 80% of the bound material was driven off by resuspending the cells in an excess of unlabeled ligand, demonstrating reversibility and suggesting that most of the bound TSP remained surface associated (and was not internalized). This is consistent with the putative functions of TSP as a surface active molecule.

The state of activation of the mononuclear cells did not appreciably effect TSP binding. Murine peritoneal macrophages stimulated by intraperitoneal thioglycollate injection before harvesting bound TSP to an equivalent extent and affinity as the resident cells (data not shown). Cells met morphologic and numerical criteria for activation. The U937 cells when stimulated by PMA also bound TSP to an equivalent extent.

It has recently been reported that purified TSP binds specifically to sulfated glycolipids (37). This binding, which is inhibited by heparin and dextran sulfate, was suggested by the authors to play a role in the lectin function of TSP. They speculated that the binding of platelet surface TSP to these lipids in the membrane of fixed erythrocytes could account for the observed agglutination. In our studies heparin did not inhibit  $^{125}$ I-TSP binding to U937 cells or macrophages (Fig. 2), nor did it inhibit platelet-U937 rosetting, suggesting that sulfated glycolipids are not the TSP binding site on these cells. The biochemical nature of this binding site is currently under investigation. We have recently obtained data identifying an 88-kD antigen on the surface of platelets, U937 cells, monocytes, and several tumor cell lines as a putative TSP receptor (45).

The presence of a high-affinity surface binding site for TSP



on the monocyte/macrophage suggests that TSP could serve an adhesive function on these cells. An interaction of PBMs and tissue macrophages with platelets is well described both as a methodologic problem in preparing "pure" populations of mononuclear cells (46) and as a clinical problem (20, 21, 47). For example, histopathologic data has shown platelets adherent to macrophages within the intima of diet-induced atherosclerosis in experimental animals (20, 21). The mechanism of the platelet-monocyte interaction has been shown by Jungi et al. (34) to be dependent upon platelet secretion and not dependent upon fibrinogen or FN. We now show that the platelet-monocyte interaction is mediated by TSP. This conclusion is based on the ability of specific antibodies (both monoclonal IgG and polyclonal Fab) to TSP to inhibit the interaction as well as the ability of purified ligand to inhibit the interaction. Controls included both nonimmune antibodies and anti-FN Fab as well as purified fibrinogen, FN and the "adhesion" tetrapeptide RGDS. Aminosugars, which inhibit the lectin function of TSP (13), did not inhibit the platelet-monocyte interaction, demonstrating that the adhesion is not a nonspecific effect of the TSP lectin. The lack of inhibition by RGDS and FN suggests that the molecular basis of TSP cytoadhesion is different from that of the FN-vitronectin-fibrinogen-vWF family. This is consistent with the recently reported observation that TSP binds normally to platelets that are genetically deficient in the glycoprotein IIb/IIIa complex (48, 49). This complex is an RGDS receptor (50) and is responsible for fibrinogen (51) and FN (52) binding to activated platelets.

The precise TSP domain responsible for the monocyte interaction is under current investigation. The monoclonal antibodies that inhibit monocyte rosette formation do not react with TSP in a Western blot assay, suggesting that they may react with an epitope(s) determined by the tertiary structure of the molecule. This epitope is probably distinct from the amino-terminal domain involved in heparin binding (28) based on the failure of heparin to inhibit TSP-monocyte binding. Monoclonal antibody C6.7, which reacts with the carboxy-terminal domain, failed to inhibit rosetting, suggesting that this region may not be involved in mediating the TSP-monocyte interaction. It is of interest that the monoclonal anti-TSP antibodies that inhibit platelet monocyte rosetting do not inhibit platelet aggregation, and vice versa.

We suggest that platelet surface-associated TSP is multivalent and can in turn bind to a specific binding site on the monocyte cell surface, creating an intracellular bridge. At sites of early vascular injury, activated platelets may thus serve to recruit and localize monocytes into an early lesion. Monocyte and macrophage surface TSP may also play a role in effecting the biological response of the cells. In particular, as we have shown in purified, in vitro systems, surface immobilized TSP can modulate heparin function (15, 16) and plasmin generation (14, 17). Both of these molecules are of critical importance in vascular biology. Heparin or heparans, for example, not only inhibit thrombin, but have other direct and indirect effect on vascular cell proliferation (53, 54). Plasminogen activator production by activated monocytes may be important in allowing penetration and migration of these cells through basement membrane (18, 55). We have suggested that surface TSP may be an important part of the regulation of plasmin generation in these circumstances. The interaction of TSP with the surface of monocytes and macrophages may play an important role in the regulation of thrombosis and the pathophysiology of atherosclerosis.

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