Thrombospondin Inhibits Adhesion of Platelets to Glass and Protein-Covered Substra
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Glass and protein-covered surfaces when treated with the platelet-secreted glycoprotein thrombospondin lose their capacity to bind unstimulated platelets. In comparison to the number that bind to fibronectin-covered glass surfaces, less than 3% bind to thrombospondin-covered glass surfaces. When the fibronectin-covered surface is incubated with thrombospondin, it loses 87% of its binding capacity for platelets. The inhibitory effect of thrombospondin on platelet binding increases with increasing amounts of the adsorbed protein and reaches maximal values at 65% saturation of the adsorption of thrombospondin to the surface. Platelet spreading on the surface is also completely inhibited by thrombospondin. These data suggest that thrombospondin is nonthrombogenic and can modulate platelet adhesion to the subendothelium.

THROMBOSPONDIN, a high–molecular weight glycoprotein, is stored in the circulating blood platelets and expressed on their surface after thrombin stimulation.1 Platelet adhesion to fibronectin- or collagen-covered surfaces also leads to expression of this protein and to its binding to the protein on the surface.2 Adhesion also leads to activation of binding sites for other α granule proteins on the platelet membrane,3 thereby enhancing platelet-matrix and platelet-platelet interactions. In suspension thrombospondin can bind to unstimulated platelets, but its binding to stimulated platelets is much enhanced.4 A specific membrane receptor for this protein has been recently described.5 These data together with the observation that purified thrombospondin can bind to cellular and plasma fibronectin,2,6,7 fibrinogen,8 and collagen2,9 and that it can self-assemble6 lead to the suggestion that it may play a role in platelet adhesion. This suggestion is further supported by the observation that a similar protein10 is also synthesized and secreted by many cell types in culture,11 it can be incorporated into the extracellular matrix,12,13 and in vivo it is present in many tissues, particularly in the interstitial space.13 A role for thrombospondin in cell adhesion has been recently invoked by the finding that in contains the sequence Arg-Gly-Asp,14 which has been shown to play a central role in the cell-adhesive properties of many proteins.15 Attachment of tumor cells to surfaces is indeed supported by thrombospondin.18,21 In view of the central role of adhesion in platelet function we undertook to study the role of thrombospondin in the initial steps of adhesion and spreading of unstimulated platelets.22

We report here our studies of the interaction of gel-filtered unstimulated human platelets with thrombospondin and of the effect of thrombospondin on the interaction of platelets with fibronectin.

MATERIALS AND METHODS

Protein. Plasma fibronectin was purified according to Engvall and Ruoslahti.24 Platelet thrombospondin, a kind gift from Dr George Adams, the Canadian Red Cross, Ottawa, was purified according to Lawler et al.25 Fatty acid–free, bovine serum albumin (BSA) was purchased from Sigma Chemical Co, St Louis. All proteins were analyzed for purity by sodium dodecyl sulfate (NaDodSO4) polyacrylamide gel electrophoresis26 after radiolabeling. Only pure and intact preparations were used.

All proteins were radiolabeled at a concentration of 0.5 to 1.0 mg/mL by using iodobeads (Bio-Rad, Richmond, CA) with Na125I (Amersham Intl, England) at 50 μCi/mL protein solution in phosphate-buffered saline containing 2 mmol/L Ca and 0.8 mmol/L Mg (PBS) according to the manufacturer’s instructions. Specific activity routinely obtained was 5 × 106 to 109 cpm/mm. The protein concentration was determined after iodination and gel filtration using the method of Lowry et al.27 Radiolabeling did not affect protein-protein interactions.3

Platelets. Fresh human blood was obtained by venipuncture from healthy volunteers who had not ingested any drugs during the ten days before bleeding. The blood was anticoagulated with 1/10 vol acid-citrate-dextrose anticoagulant (85 mmol/L trisodium citrate, 65 mmol/L citric acid, 2% dextrose) and processed within one hour of withdrawal. The platelets were separated from other blood cells and proteins by differential centrifugation at 120 g for 20 minutes followed by gel filtration through Sepharose 2B-CL (Pharmacy Fine Chemicals, Piscataway, NJ) as described earlier.2 Briefly, the column was pre-equilibrated with Lindon’s buffer28 containing 129 mmol/L NaCl, 8.9 mmol/L NaHCO3, 2.8 mmol/L KCl, 0.8 mmol/L KH2PO4, 0.8 mmol/L MgCl2, 2.4 mmol/L CaCl2, 10.9 mmol/L Na Citrate, 2 H2O, 1 mg/mL glucose, and 10 mmol/L Tris, pH 7.3, and the platelets were eluted with the same buffer. The platelet concentration was determined by counting in a hemocytometer and adjusted to be 109 platelets/mL.

Assays. Round glass coverslips were incubated at room temperature in 24-well plates (Nunc Roskilde, Denmark) with 300 μL protein solution in PBS. For the determination of time dependence of protein adsorption, 100 μg/mL protein was used with iodinated protein added as a tracer. Coverslips were removed at the appropriate times, rinsed thoroughly in PBS, and counted in Beckman Gamma Counter (Beckman Instruments, Inc, Fullerton, CA). For determination of the concentration dependence of adsorption, protein in PBS at a concentration range of 5 to 80 μg/mL with fixed amounts of iodinated protein added as a tracer was incubated in the wells for three hours and the coverslips rinsed and counted.

Coating of coverslips for platelet adhesion studies was done by incubation in protein solution for three hours at room temperature followed by rinsing in the buffer in which the cells were suspended. Saturation of the surface with adsorbed protein was obtained with
100 μg/mL protein. Subsaturating coating with thrombospondin was obtained with 5 to 80 μg/mL protein. Sequential coating with two proteins was achieved by following the first coating with thorough rinsing and incubation in 100 μg/mL of the second protein for another three hours at room temperature.

The determination of bound platelets was done by incubating 0.3 mL gel-filtered platelets (10⁷ platelets/mL unless noted otherwise) in each well for 45 minutes at room temperature followed by removal of the coverslips from the wells and repeatedly dipping them in glasses filled with Lindon’s buffer, while held by forceps, for thorough rinsing. The adhering platelets were then counted by direct visualization in phase-contrast using a Leitz Ortholux microscope. A 40× objective was used with a 10× eyepiece with a reticulum. Six fields were counted on each coverslip, and there were three coverslips per protein.

RESULTS

Adsorption of thrombospondin, fibronectin, and albumin to glass. Study of the kinetics of adsorption showed that the rate and extent of adsorption varied greatly from protein to protein (Fig 1). Plateau values were reached for the slowest protein in the group—fibronectin—after 180 minutes. This incubation time was therefore used in all subsequent experiments to ensure maximal adsorption. The concentration dependence of adsorption (Fig 2) demonstrated a typical Langmuir-type isotherm. The amount of adsorbed thrombospondin was similar to that of fibronectin and more than three times higher than that of albumin. The different proteins achieved maximal adsorption at different concentrations, but all reached plateaus at less than 100 μg/mL. Therefore in all subsequent experiments coating of cover slips to saturation was done with 100 μg/mL protein solutions.

Adhesion of platelets to protein-covered substrata. (a) Gel-filtered platelets suspended in medium containing Ca²⁺ and Mg²⁺ at the same concentration as in citrated blood adhered without aggregation to glass surfaces and to surfaces covered to saturation with protein. The extent of binding greatly depended on the protein on the substratum. Of the different substrata compared, the highest number of platelets adhered to fibronectin, 6,900 ± 1,020 platelets/mm² (Fig 3). In sharp contrast, thrombospondin virtually inhibited platelet binding, bringing it down to 200 ± 100 platelets/mm², which is less than 3% of the number that bound to fibronectin (Fig 3). Albumin showed some capacity for platelet adhesion (830 ± 90 platelets/mm², which is 12% of the number that bound to fibronectin), but as expected, it was almost three times lower than the adhesive capacity of glass (2,000 ± 1,100 platelets/mm²) (Fig 3). A tenfold increase in platelet concentration in the bulk caused a nonlinear increase in platelet adhesion. Although binding to fibronectin increased fivefold, binding to thrombospondin increased 25-fold. Binding to albumin showed a tenfold increase as expected (Fig 4). (b) The concentration dependence of the inhibitory effect of thrombospondin on platelet adhesion was tested on glass surfaces that had been incubated with a series of subsaturating concentrations of thrombospondin in the range of 5 to 80 μg/mL. When equal numbers of platelets were applied to these surfaces, we found that, with increasing thrombospondin coverage, platelet adhesion fell by 90% (Fig 5). Minimal binding was reached at 65% of the saturation level of surface-attached thrombospondin as seen by comparing Figs 1 and 5. (c) Sequential incubation of the glass surface with different proteins yielded composite substrata that were then tested for their platelet adhesiveness. The determination of bound platelets was done by incubating 0.3 mL gel-filtered platelets (10⁷ platelets/mL unless noted otherwise) in each well for 45 minutes at room temperature followed by removal of the coverslips from the wells and repeatedly dipping them in glasses filled with Lindon’s buffer, while held by forceps, for thorough rinsing. The adhering platelets were then counted by direct visualization in phase-contrast using a Leitz Ortholux microscope. A 40× objective was used with a 10× eyepiece with a reticulum. Six fields were counted on each coverslip, and there were three coverslips per protein.

![Fig 1](image1.png) Time dependence of adsorption of thrombospondin (△), fibronectin (○), and albumin (●) to glass at room temperature. The protein concentration was 100 μg/mL; two adsorption experiments are shown for each protein.

![Fig 2](image2.png) Concentration dependence of adsorption of thrombospondin (○), fibronectin (●), and albumin (●) to glass at room temperature. The time of incubation was three hours. Each point represents the mean of the amounts adsorbed to three glass coverslips. The SD was in all cases <6%.

![Fig 3](image3.png) Platelet binding to protein-covered and glass surfaces: TSP, thrombospondin; FN, fibronectin; BSA, bovine serum albumin–covered glass surface; Gls, glass not precovered by protein.
adhesion capacity. The fibronectin-covered surface, when further incubated with thrombospondin, lost 87% of its binding capacity (Fig 6). Conversely, the thrombospondin-covered surface incubated with fibronectin increased its binding capacity fivefold (Fig 6). Albumin-covered surfaces, when incubated with thrombospondin or fibronectin, showed a similar although much less pronounced effect: thrombospondin inhibited platelet binding per unit area by 30%, and fibronectin increased it by 25%.

**Spreading of platelets.** Spreading of the platelets also depended on the identity of the protein covering the surface. On thrombospondin, platelets did not spread at all but stayed completely round for up to 90 minutes (Fig 7, top). On fibronectin, on the other hand, they were completely spread within 30 minutes of incubation with the surface (Fig 7, bottom).

**DISCUSSION**

We find that thrombospondin inhibits the adhesion of unstimulated platelets to substrata, both when it is directly adsorbed to glass and when it is bound to fibronectin.

Tuszynski et al recently reported that the number of platelets that bind to thrombospondin adsorbed to glass is similar to that which binds to fibronectin. This apparent discrepancy between our observation and that of Tuszynski et al stems from the fact that the number of platelets that bind to fibronectin in our hands, as has been previously reported, is routinely ten times higher than that reported by Tuszynski et al, whereas the number of platelets that bind to thrombospondin is similar at similar platelet concentrations. The cause for the difference in binding to fibronectin is not known and can stem from differences in the method of preparation of the fibronectin; drying of the protein droplet on the coverslip, which will lead to its denaturation; or other technical differences. The difference in binding to albumin...
that was also observed could stem from the use of different preparations of this protein. Thrombospondin secreted either by endothelial cells or by the blood platelet after stimulation can bind to purified fibronectin. We show here that in this complex it can also inhibit platelet adhesion and spreading. Because fibronectin is a matrix protein, it is suggested that the two proteins form a complex in the matrix. Indeed, thrombospondin was detected in the matrix of bovine aortic endothelial cells, and it codistributes there with fibronectin (M. Dreyfus and J. Lahav, submitted). Our results therefore indicate that thrombospondin inhibits the adhesion of unstimulated platelets to the subendothelium. A platelet receptor for thrombospondin has been recently identified, but when on the intact platelet it is only active after platelet stimulation, much like the fibrinogen receptor. It has been reported that in suspension the unstimulated platelet can bind small amounts of thrombospondin to its membrane. It is possible that this capacity is not manifested in the interaction of the unstimulated platelet with surface-adsorbed thrombospondin because the affinity and the number of binding sites on the unstimulated platelet are too small to support adhesion to the surface-adsorbed thrombospondin and an increase in those parameters such as occurs after platelet stimulation will turn the surface more adhesive. Another possible explanation is that adsorption of thrombospondin to glass or to fibronectin masks the binding domains of thrombospondin. This will imply that complexes of thrombospondin such as exist in the extracellular matrix decrease the thrombogenicity of the matrix. Both explanations suggest that before platelet activation thrombospondin is nonthrombogenic and will inhibit platelet adhesion.

REFERENCES

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