

Glycoprotein IV Mediates Thrombospondin-dependent Platelet-Monocyte and Platelet-U937 Cell Adhesion

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Abstract

An adhesive interaction between activated platelets and mononuclear phagocytes may contribute to the role these cells play in regulating inflammation, thrombosis, and atherosclerosis. We have previously shown that this adhesive interaction is mediated by the expression of the glycoprotein thrombospondin (TSP) on the surface of activated platelets. We now show that TSP-dependent platelet-monocyte interactions are mediated by glycoprotein IV (GPIV), an intrinsic membrane protein recently identified as a cell surface TSP receptor. Monoclonal antibodies to GPIV bound to cells of the human monocytoid line U937 as assessed by flow cytometry and inhibited the binding of ^{125}I -TSP to the cell surface by 83%. U937 cells preincubated with anti-GPIV were not rosetted by thrombin-stimulated platelets (72% inhibition compared with control anti-monocyte antibodies). In addition, when platelets were stimulated in the presence of saturating concentrations of monoclonal antibodies to GPIV, only 18% of U937 cells were rosetted (78% inhibition). Control antibodies including anti-GPIb did not inhibit rosette formation. These data suggest that TSP can cross-link platelets and monocytes via an interaction with GPIV on the surface of both cells. This molecular bridge may mediate platelet-macrophage communication in various pathophysiological settings.

Introduction

Cell-cell interactions may play a critical role in regulating the molecular events that occur during inflammation, thrombosis, and atherosclerosis. In particular, an adhesive interaction of activated platelets with mononuclear phagocytes at sites of vascular injury has been shown to be one of the earliest morphologically recognized events in experimental atherosclerosis (1). In addition, platelets have been shown to influence macrophage activation and facilitate tissue factor induction (2) while macrophage thromboxane has been shown to potentiate platelet aggregation (3). Jungi et al. (4) have recently described a model system in which thrombin-stimulated human platelets specifically form rosettes with U937 cells (a monocytoid human cell line [5]) and human monocytes. They found this adhesive interaction to be dependent upon platelet secretion but not upon fibrinogen or fibronectin. We subsequently dem-

onstrated that the adhesive platelet glycoprotein thrombospondin (TSP)¹ binds specifically and with high affinity to mononuclear phagocytes and that platelet surface TSP mediates this platelet-monocyte interaction (6). Inhibition studies with both antibodies to TSP and excess TSP suggested that rosette formation was dependent upon the interaction of platelet surface TSP with an unidentified receptor on the monocyte surface (6).

The role of TSP as an adhesive protein and the precise mechanisms of TSP binding to cell surfaces are not yet fully defined. TSP has been shown to mediate cell-cell interactions in several systems in addition to platelet-monocyte adhesion. On the platelet surface, for example, TSP promotes platelet aggregation (7-9), and TSP or TSP-like molecules may be involved in the adherence of falciparum malaria-infected red blood cells to endothelium (10). In addition, several normal and tumor cell lines have been shown to attach and spread on immobilized TSP (11-13).

The molecular basis of TSP-cell interactions is complex. Although TSP contains within its primary structure the adhesion sequence RGDA (14, 15), we have shown that the tetrapeptide RGDS did not inhibit platelet-monocyte adhesion (6). Similarly TSP did not bind to the purified platelet integrin receptor glycoprotein (GP)IIb/IIIa (16), but did bind normally to platelets genetically deficient in GPIIb/IIIa (17). Certain monoclonal antibodies to GPIIb/IIIa, however, inhibited TSP binding to platelets (18), suggesting that GPIIb/IIIa itself or GPIIb/IIIa-associated fibrinogen or fibronectin may play a secondary role in TSP-platelet interactions. Thus TSP-platelet interactions seem to be mediated by receptor-ligand interactions different from those of fibronectin, fibrinogen, and vitronectin.

TSP also binds specifically to heparin (19) and sulfated glycolipids (20). Exogenous heparin effectively inhibited fibroblast and endothelial cell TSP binding and internalization (21, 22), suggesting that the heparin-binding domain of TSP may mediate this type of cell-TSP interaction perhaps through cell surface-associated heparin-like molecules. Similarly heparin inhibited the spreading of some tumor cells on immobilized TSP (12). We have previously shown, however, that heparin did not inhibit platelet-monocyte rosette formation or TSP binding to monocytes.

In this paper we identify the monocyte surface TSP receptor immunologically as glycoprotein IV (GPIV). This intrinsic 88-kD, single chain, heavily glycosylated membrane protein, first described in platelets (23-25), is also known as GPIIb and CD36 and is the antigen recognized by the commercially avail-

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1. Abbreviations used in this paper: GP, glycoprotein; TSP, thrombospondin.

able murine monoclonal antibody OKM5 (26, 27). This antibody reacts with platelets, monocytes, some endothelial cells (28), and a variety of tumor cell lines including melanoma C32 and fibrosarcoma HT1080. Studies from this laboratory (29) recently characterized GPIV as a TSP receptor based on the ability of OKM5 to inhibit platelet surface TSP expression and tumor cell TSP binding, as well as by demonstrating specific complex formation of purified TSP with purified GPIV. Monoclonal antibodies to GPIV, however, did not inhibit attachment and spreading of other melanoma lines on TSP (12) nor did they inhibit endothelial cell TSP uptake and degradation (Mosher, D., personal communication).

Methods

Purified proteins. Purified human calcium-replete TSP was prepared from ionophore A23187-induced platelet releasate as previously described (30) and was labeled with ^{125}I by the modified chloramine-T method to a specific activity of 10^7 cpm/mg. Bovine serum albumin (Pentex BSA) was obtained from Miles Laboratories, Inc., Naperville, IL, and human thrombin was a gift of Dr. J. Fenton II, New York State Department of Health, Albany, NY.

Antibodies. Normal rabbit and mouse IgG were purchased from Cappel Laboratories, Cochranville, PA. Affinity-purified fluorescein-conjugated goat anti-mouse IgG was from Tago Inc., Burlingame, CA. Anti-GPIV antibodies were obtained from the following sources: OKM5 was a gift of Dr. G. Goldstein (Ortho Diagnostics, Westwood, MA), 1B1G7, 8A6D11 and rabbit anti-C32 melanoma-derived GPIV were provided by Dr. J. Barnwell (New York University, Medical Center, New York, NY). 1B1G7, 8A6D11, and the rabbit anti-GPIV antiserum were all raised by immunizing animals with purified C32-derived 88-kD antigen prepared by affinity chromatography of cell lysates on a column of OKM5 IgG-Sepharose. These antibodies did not react with TSP by ELISA, and all immunoprecipitated a single ~ 88-kD protein from monocytes (26) or C32 melanoma cells (29, Barnwell, J., personal communication). Control monoclonal anti-monocyte antibodies OKM6 (reactive with a 116-kD monocyte surface protein), 3G8 (reactive with monocyte Fc receptor), and anti-LeuM3 (reactive with CD14, a 55-kD monocyte surface protein) were from Ortho Diagnostics, Dr. J. Unkeless (Mt. Sinai Medical Center, New York), and Becton, Dickinson & Co. (Mountain View, CA), respectively. AP1 (anti-platelet GPIb) was from Dr. T. Kunicki (Milwaukee, WI).

Cells and cell culture. U937 cells were maintained as previously described (6) 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-cm² flasks. Human PBMC were isolated from dilute citrated whole blood as previously described (6). Human platelets were isolated from fresh citrated platelet-rich plasma by gel filtration on a column of Sepharose 2B (Pharmacia Fine Chemicals, Piscataway, NJ) as previously described (6). Platelets were pooled and stimulated under dilute nonstirring conditions with human thrombin (0.2 U/ml) 22°C. After 15 min D-phenylalanyl-L-prolyl-arginine chloromethyl ketone 10^{-6} M was added for 3 min and then the platelets were lightly fixed with 1.1% formaldehyde at 22°C for 60 min according to the method of Jungi et al. (4). The fixed platelets were then washed three times and resuspended in buffer containing 2 mg/ml BSA and were used within 24 h of preparation. In some studies the platelets were stimulated either in the presence of monoclonal antibodies to GPIV or control antibodies.

Immunofluorescence flow cytometry. Washed U937 cells were resuspended in PBS containing 2% BSA and 2% goat serum at $1.8 \times 10^7/\text{ml}$ and 0.1 ml was incubated with monoclonal antibodies (10 $\mu\text{g}/\text{ml}$) for 1 h at 22°C. The cells were then washed three times and

resuspended in FITC goat anti-mouse IgG in the same buffer for 1 h at 22°C. After repeat washing the cells were resuspended in filtered PBS and immediately analyzed using a flow cytometer equipped with a MDADS data handling computer and an argon laser (Epics; Coulter Instruments, Inc., Hialeah, FL). A wide sizing gate was set by examination of both right angle and forward angle light scatter signals from nonstained cells to include all nucleated cells. For each sample 10^4 cells were counted within this gate.

Immunoprecipitation. U937 cells (9×10^7) were washed, suspended in 1 ml of PBS, and surface-labeled with 1 mCi ^{125}I -NaI for 20 min at 20°C using *N*-chloro-benzenesulfonamide-derivatized polystyrene beads (Iodo-Beads; Pierce Chemical Co., Rockford, IL). The cells were then washed four times in cold PBS and lysed in 1 ml of 20 mM Tris, 100 mM NaCl, 1% NP40, 1 mM PMSF, 1 mM NEM, 1 mM leupeptin, pH 7.2, for 1 h at 4°C. The cytoskeletal and nuclear debris were removed by centrifugation and the lysates cleared by incubation with either goat anti-mouse IgG-agarose beads (Bio-Rad Laboratories, Richmond, CA) or *Staphylococcus aureus* protein A-agarose beads (Sigma Chemical Co., St. Louis, MO) for 1 h at 4°C. The lysates were then incubated with either monoclonal anti-GPIV IgG (8A6D11, 1B1G7, OKM5), rabbit anti-GPIV IgG, or nonimmune IgG for 16 h at 4°C. Antigen-antibody complexes were removed by incubation with either goat anti-mouse-agarose beads or protein A-agarose beads that had previously been cleared with nonlabeled U937 lysates. The beads were then extensively washed in lysis buffer containing 0.1% NP40 before resuspension in SDS-PAGE sample buffer. These were boiled for 2 min before electrophoresis under nonreducing conditions on 7.5% SDS-PAGE gels. The gels were fixed and dried, and autoradiographs were obtained using XAR-5 film (Eastman Kodak Co., Rochester, NY).

^{125}I -TSP binding assays. Binding of TSP to U937 cells was quantified as described previously (6). U937 cells were washed three times, resuspended at 2×10^6 cells/ml, and then incubated in serum-free medium made up of RPMI 1640 containing 2 mg/ml BSA (RPMI/BSA) for 1 h before use. The cells were then rewashed and aliquoted into 1.5-ml polypropylene microcentrifuge tubes ($1.5\text{--}2.0 \times 10^6$ cells per aliquot, 0.1 ml per tube). To these was added 50 μl of radioiodinated TSP (175 nM) diluted also in RPMI/BSA containing either OKM5 (10 $\mu\text{g}/\text{ml}$), control anti-monocyte antibodies or EDTA (5 mM). After incubation at 4°C for 1 h the cell suspensions were layered onto 0.4 ml of silicone oil (67:33 vol/vol of No. 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 and oil were removed and then the tubes were inverted and the tips containing the cell pellet were amputated and placed in a gamma counter to assess cell-bound radioactivity.

Platelet-monocyte adhesion assay. Platelet-U937 and platelet-monocyte interactions were quantified as previously described (6) with a rosetting assay modified after the method of Jungi et al. (4). 50 μl of gel-filtered, thrombin-stimulated, fixed platelets ($3\text{--}5 \times 10^7$ platelets) were incubated with 100 μl of U937 cells or peripheral blood mononuclear cells ($3\text{--}5 \times 10^5$ cells) rotating end-over-end in a 12 \times 75-mm polypropylene tube at 4°C. The cells were then placed in a Neubaur chamber and examined by phase-contrast light microscopy. Platelet adhesion was quantified by counting at least 200 U937 cells or monocytes 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 platelets, the mononuclear cells were preincubated for 30 min with monoclonal anti-GPIV antibodies (10 $\mu\text{g}/\text{ml}$) or control antibodies.

Results

U937 cells express GPIV on their surfaces. Indirect fluorescence flow cytometry using murine monoclonal anti-GPIV IgG (OKM5) and FITC-conjugated goat anti-mouse IgG was

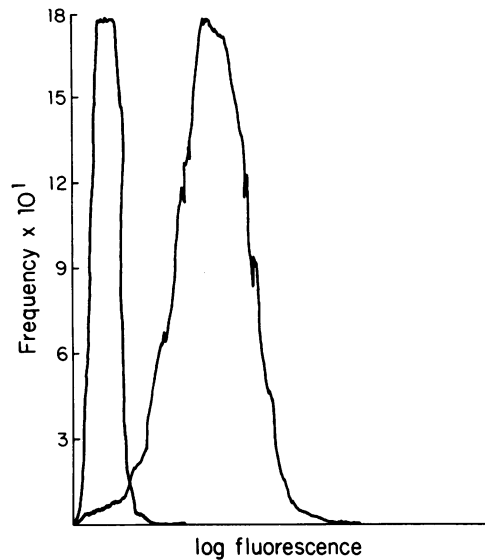


Figure 1. Surface expression of GPIV by U937 cells. Cells of the human monocytoid line U937 were incubated with 10 $\mu\text{g}/\text{ml}$ murine monoclonal anti-GPIV (OKM5) or with isotype-matched nonimmune mouse IgG for 1 h at 22°C. After washing, the cells were incubated with FITC goat anti-mouse IgG for another hour, washed, and resuspended in filtered PBS, and surface fluorescence was analyzed by flow cytometry. The tracing shows that < 2% of the cells reacted with control antibody (left tracing) whereas > 95% reacted with OKM5.

used to demonstrate that U937 cells express GPIV on their surfaces. Although U937 cells were reported in the initial characterization of OKM5 not to express this antigen unless stimulated by phorbol esters (26), we now show, as seen in Fig. 1, that > 95% of the cells bound OKM5 whereas < 2% bound an

isotype-matched control murine IgG. Similar fluorescence patterns were seen with the other monoclonal and polyclonal anti-GPIV IgG's. Immunoprecipitation studies using monoclonal and polyclonal anti-GPIV IgG were carried out to examine the biochemical nature of this U937 OKM5-reactive antigen. As shown in Fig. 2, the rabbit polyclonal anti-GPIV (lane A) and the three murine monoclonal anti-GPIV antibodies (lanes C-E) specifically precipitated a single protein of ~ 88-kD from ^{125}I surface-labeled cells. This broad band of reactivity is very similar to that seen by Talle et al. (26) with OKM5 immunoprecipitates of human peripheral blood monocytes. This finding suggests that U937 cell GPIV is structurally similar to monocyte GPIV.

^{125}I -TSP binding to U937 cells is inhibited by OKM5. In a fluid-phase suspension binding assay (Fig. 3) OKM5 at saturating concentrations (10 $\mu\text{g}/\text{ml}$) inhibited 82 \pm 9% of total binding of ^{125}I -TSP to the U937 cells. Binding was carried out as previously described at 4°C under equilibrium conditions at a TSP concentration approximately equal to the K_d (175 nM). Specific binding (defined as that not inhibited by divalent cation chelation) represented 91 \pm 9% of the total binding in these experiments. Control anti-monocyte monoclonal antibodies anti-LeuM3 and 3G8 did not inhibit TSP binding. 20-fold molar excesses of human albumin, plasminogen, or gamma globulin did not inhibit ^{125}I -TSP binding to these cells while previous studies demonstrated > 90% inhibition of specific binding by unlabeled TSP in a 20-fold molar excess (6). Heparinized human plasma (final concentration 50%) or 20–40-fold excess fibrinogen partially inhibited binding (mean 30% inhibition in three experiments) suggesting that TSP-protein interactions can modulate TSP-monocyte binding. Treatment of the U937 cells with trypsin (250 $\mu\text{g}/\text{ml}$) for 30 min at 37°C also did not inhibit TSP binding. This is consistent with previous reports that platelet surface GPIV is resistant to trypsin digestion (24).

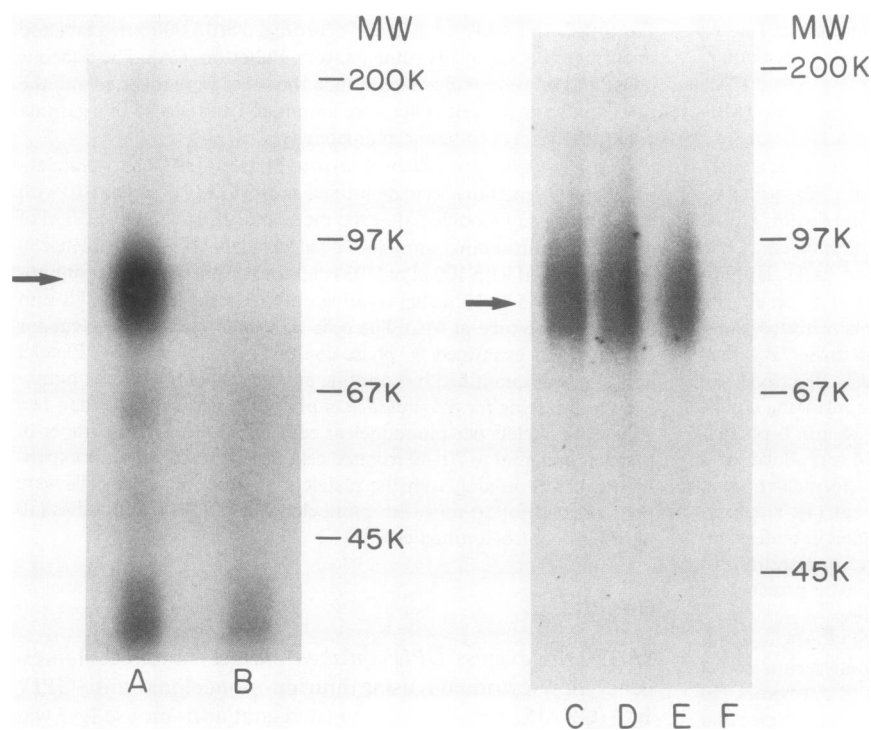


Figure 2. Immunoprecipitation of GPIV from U937 cells. ^{125}I -labeled surface proteins from U937 cells were extracted with 1% NP40 and the extracts were cleared of nonspecifically adhering material with nonimmune IgG and either protein A-agarose beads or goat anti-mouse IgG beads. Specific antigens were then precipitated using either rabbit anti-GPIV IgG + protein A beads (lane A), mouse monoclonal anti-GPIV 8A6D11 (lane C), 1B1G7 (lane D), or OKM5 (lane E) + goat anti-mouse IgG beads. Lanes B and F are control precipitates using nonimmune rabbit IgG (lane B) or mouse IgG (lane D). After washing, the beads were boiled in SDS-PAGE sample buffer, electrophoresed under nonreducing conditions on 7.5% SDS-PAGE gels, and autoradiographs obtained. Molecular weight (MW) determinations (K, thousands) were made from pre-stained markers run on the same gel.

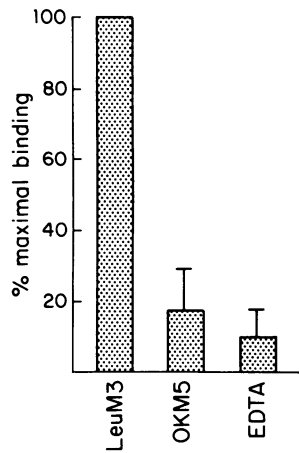


Figure 3. Monoclonal anti-GPIV inhibits ^{125}I -TSP binding to U937 cells. U937 cells (2×10^6) were incubated with ^{125}I -TSP (175 nM; 10^7 cpm/mg) in RPMI/BSA containing either control antibody (anti-LeuM3), OKM5 (10 $\mu\text{g}/\text{ml}$), or 5 mM EDTA for 1 h rotating at 4°C. Bound and free ligands were then separated by centrifugation (14,000 g for 5 min) through silicone oil and radioactivity was counted. Bars represent the mean \pm SD of four experiments expressed as percentage of total binding in the absence of antibody.

Monoclonal antibodies to GPIV inhibit platelet-U937 and platelet-monocyte rosette formation. Previous studies have shown that the specific rosetting of peripheral blood mono-

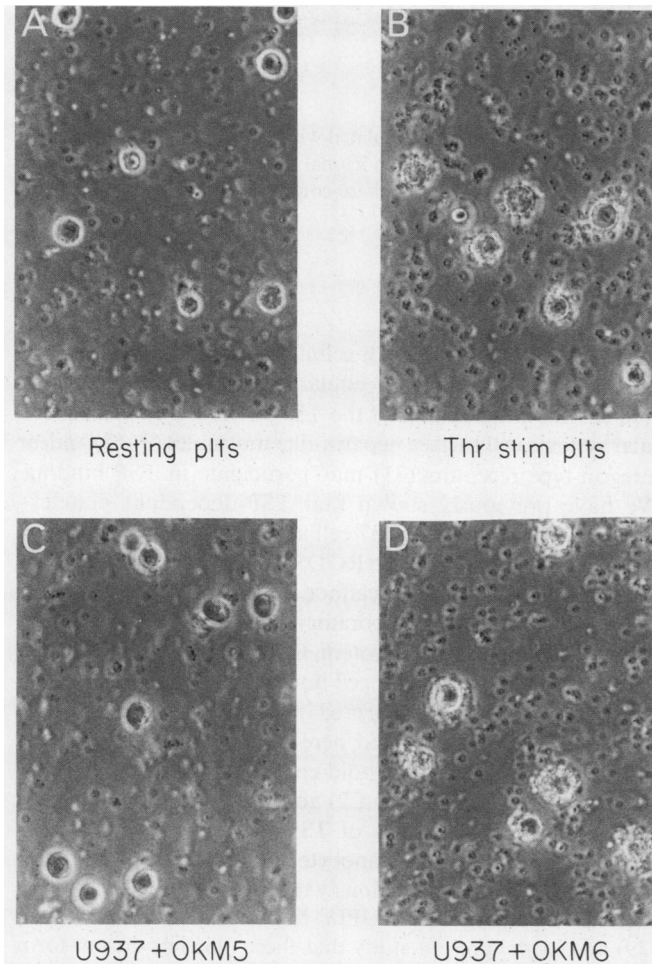


Figure 4. Anti-GPIV antibodies inhibit platelet-U937 rosette formation. (A) Fixed resting platelets or (B-D) fixed thrombin-stimulated platelets were incubated for 30 min rotating end-over-end at 4°C with U937 cells and fresh wet mounts examined by phase-contrast microscopy ($\times 769$). (C) Experiment in which the U937 cells were incubated with OKM5 (10 $\mu\text{g}/\text{ml}$) for 15 min before the addition of platelets and (D) with control anti-monocyte IgG.

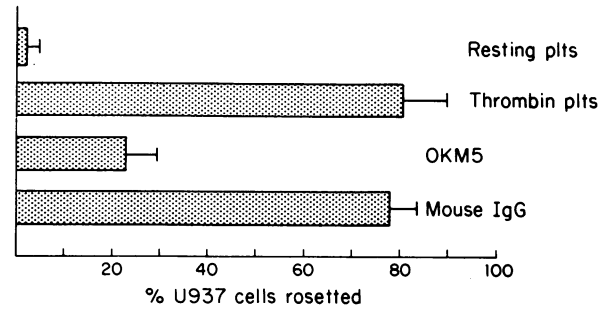


Figure 5. Role of U937 GPIV in mediating platelet-U937 cell interactions. U937 cells and platelets were incubated as in Fig. 4, placed in a Neubaur chamber, and examined under phase contrast. 200 U937 cells were scored for presence of platelet rosetting (defined as more than two associated platelets per cell). Data are expressed as mean \pm SD of at least three separate experiments.

cytes or cells of the human monocytoïd line U937 by thrombin-stimulated human platelets is mediated by the interaction of platelet surface TSP with an unidentified receptor on the monocyte surface (6). Fig. 4, A and B, shows a typical rosetting study and demonstrates that thrombin-stimulated platelets but not resting platelets form rosettes around U937 cells. Fig. 4 C shows that just as U937 cells preincubated with OKM5 are incapable of binding ^{125}I -TSP they are also not rosetted by thrombin-stimulated platelets. Nonimmune mouse IgG or control anti-monocyte antibodies did not inhibit rosette formation (Fig. 4 D). Quantitative analysis of these studies (Fig. 5) revealed that $76 \pm 8\%$ of U937 cells were rosetted in the presence of control antibodies whereas $23 \pm 5\%$ were rosetted in the presence of OKM5 (72% inhibition). Similar results were obtained with the other monoclonal anti-GPIV antibodies and when peripheral blood monocytes were substituted for U937 cells (Fig. 6). In the presence of control anti-monocyte antibodies $73 \pm 11\%$ of the monocytes were rosetted by thrombin-stimulated platelets, whereas $20 \pm 9\%$ were rosetted in the presence of OKM5. These data suggest that the platelet "receptor" on monocytes and U937 cells is GPIV.

The ligand for this "receptor" as shown previously is platelet surface-associated TSP. Consistent with this conclusion are data from multiple investigators showing that thrombin-stimulated platelets express surface TSP while resting platelets express little or no TSP (18), and our observations that antibod-

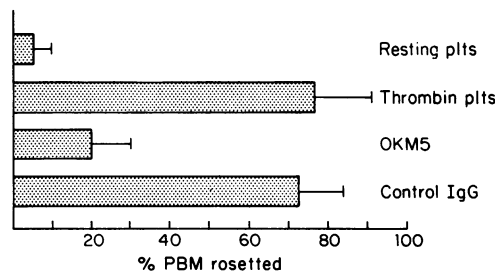


Figure 6. Role of monocyte GPIV in mediating platelet-monocyte cell interactions. Human peripheral blood monocytes were incubated as in Fig. 4, and scored for presence of platelet rosetting as in Fig. 5. Data are expressed as mean \pm SD of three experiments.

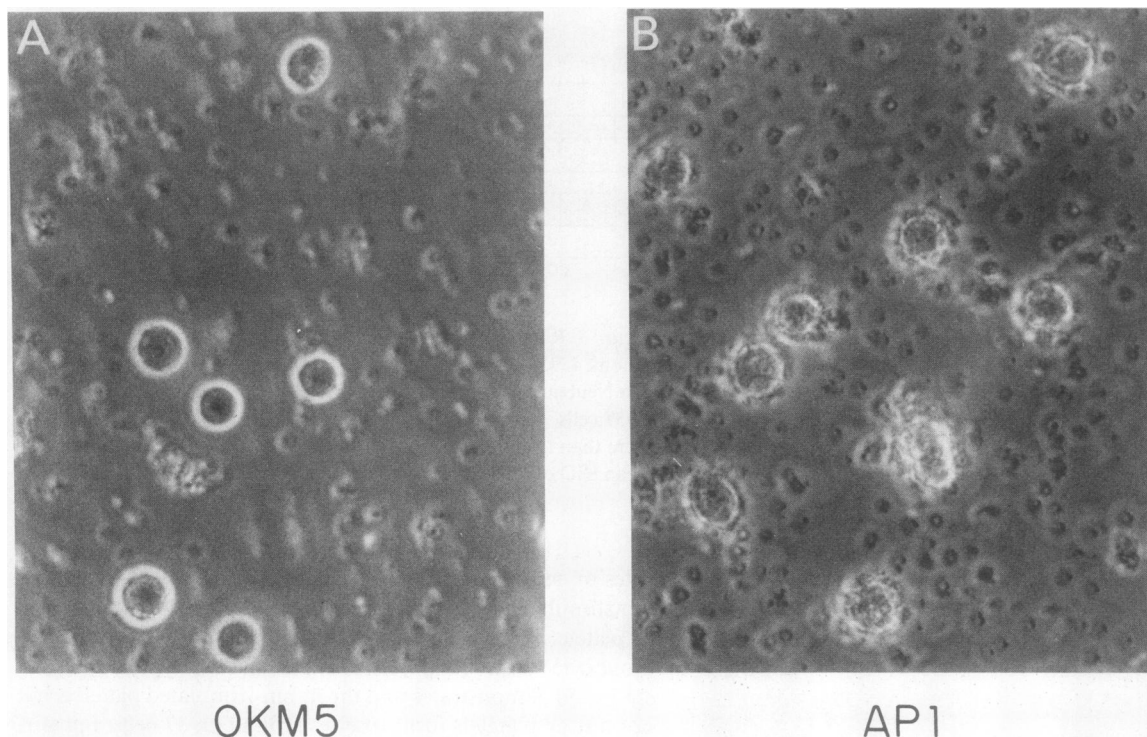


Figure 7. Platelets stimulated in the presence of anti-GPIV do not rosette U937 cells. Platelets were stimulated with thrombin (0.1 U/ml) under nonstirring conditions in the presence of (A) monoclonal anti-GPIV (OKM5 10 $\mu\text{g/ml}$) or (B) monoclonal anti-GPIb (AP1 20 $\mu\text{g/ml}$), fixed in formaldehyde, and incubated with U937 cells as in Fig. 4. Fresh wet mounts were examined by phase-contrast microscopy ($\times 1,000$).

ies to TSP as well as excess fluid-phase TSP inhibited rosette formation (6). We have previously shown that platelet surface TSP expression is inhibited when the platelets were stimulated in the presence of saturating concentrations of OKM5 (29). Fig. 7 A shows that these platelets do not rosette U937 cells whereas platelets stimulated in the presence of AP1 (monoclonal anti-GPIb) rosette normally (Fig. 7 B). Quantitation of these studies (Fig. 8) revealed $13 \pm 4\%$ and $18 \pm 6\%$ rosetting by platelets stimulated in the presence of monoclonal anti-GPIV IgG (OKM5 and 1B1G7, respectively) compared with $72 \pm 8\%$ when stimulation was in the presence of AP1 and $78 \pm 4\%$ in the presence of nonimmune mouse IgG.

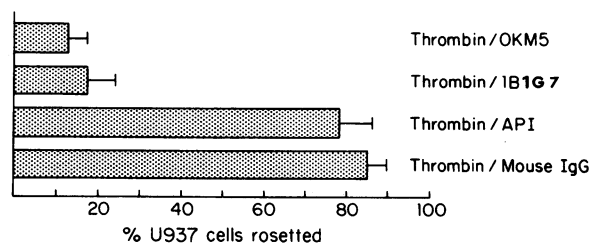


Figure 8. Role of platelet GPIV in platelet-U937 rosette formation. U937 cells and platelets were incubated as in Fig. 7 with either monoclonal antibodies to GPIV (OKM5 or 1B1G7) or controls (monoclonal anti-GPIb or nonimmune mouse IgG), placed in a Neubaur chamber, and examined under phase contrast and were scored for presence of platelet rosetting. Data are expressed as mean \pm SD of at least three separate experiments.

Discussion

The interaction of TSP with cellular surfaces is complex and probably involves multiple cellular binding sites (receptors) as well as multiple domains of the TSP molecule. In certain cellular systems cell surface heparin-like molecules (9–12) and/or integrin-type receptors (31) may participate in TSP binding. We have previously shown that TSP-dependent platelet-monocyte and platelet-U937 cell adhesion is not inhibited by the integrin-binding peptide RGDS or by heparin (6), suggesting that these mechanisms cannot explain TSP binding to the monocyte surface. This laboratory has recently identified an intrinsic membrane glycoprotein, GPIV, as a TSP receptor on platelets and on two tumor cell lines (C32 and HT1080) (29). This has recently been confirmed in studies by McGregor et al. (27). In the studies reported here we now demonstrate that cells of the human monocytoïd cell line U937 express GPIV on their surface (Figs. 1 and 2) and that antibodies to GPIV inhibit both direct binding of TSP to the cells (Fig. 3) and TSP-dependent platelet-monocyte adhesion (Fig. 4–6). In addition, we have shown previously that platelets activated in the presence of antibodies to GPIV do not express surface TSP (29) and in the present study that these platelets do not form rosettes with U937 cells (Figs. 7 and 8). The data thus suggest that TSP is multivalent and can form a bridge linking platelets and U937 cells. TSP secreted by platelets becomes bound to the platelet surface via GPIV (with perhaps some additional interactions with fibrinogen and/or GPIIb/IIIa) and then binds to a previously unoccupied GPIV molecule on the monocyte resulting in an adhesive interaction.

TSP bridging of platelets and monocytes may have important pathophysiological consequences, for example, by recruiting monocytes into an area of early vascular injury an array of proteases, cytokines, and growth factors will be presented to the vessel wall. In addition each of the cells may potentiate the activity of the other (2, 3) leading to an amplification of the injury response. Since TSP is capable of binding specifically to a number of biologically active macromolecules (32) including heparin (19), fibrinogen (16), plasminogen (33), plasminogen activator (34), and histidine-rich glycoprotein (35), its binding to GPIV on the monocyte/macrophage cell surface may also serve to localize these macromolecules to the area of injury, further contributing to the regulation of thrombosis and pathophysiology of atherosclerosis.

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