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Interaction of Thrombin-Stimulated Platelets with Vitronectin (S-Protein of Complement) Substrate: Inhibition by a Monoclonal Antibody to Glycoprotein IIb-IIIa Complex

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Key words

Platelet adhesion – Vitronectin – Glycoprotein IIb and IIIa

Summary

Platelets adhere to vitronectin substrate following activation with physiological concentrations of thrombin. Adhesion of activated platelets to vitronectin substrate is dependent upon the presence of divalent cations, the amount of vitronectin, and the duration of adhesion assay. The adhesion of platelets is inhibited by synthetic peptides containing the sequence of Arg-Gly-Asp. In addition, monoclonal antibodies to glycoprotein IIb-IIIa complex inhibit the adhesion of activated platelets to vitronectin substrate in a dose-dependent manner. These studies suggest that the glycoprotein IIb-IIIa complex on activated platelets may interact with vitronectin substrate through the Arg-Gly-Asp mechanism. Since vitronectin is present in the subendothelial matrix, it might be involved in platelet-vessel wall interactions.

Introduction

Vitronectin, also known as serum spreading factor, is a glycoprotein involved in the adhesion of a variety of cells to matrix components through the Arg-Gly-Asp sequence (2, 12, 13, 23, 26). It is identical to the S protein of the complement cascade, which inhibits complements mediated cell lysis (15, 21, 30). It is present at a relatively high concentration, 100 μg/ml in plasma (5). Using monoclonal antibodies, it has been shown that vitronectin is associated with platelets among blood cells (2, 13). Liposomes containing platelet glycoproteins IIb and IIIa bound to vitronectin substrates by the Arg-Gly-Asp dependent mechanism (23). We recently reported the thrombin-induced divalent cation dependent binding of ^{125}I-labelled vitronectin in solution to intact platelets and the inhibition of this binding by a monoclonal antibody to platelet glycoprotein IIb–IIIa complex (13). In this communication we have examined, whether surface bound vitronectin can serve as a substrate for platelet adhesion under resting and following stimulation. We have also studied the effects of peptides containing the Arg-Gly-Asp sequence and a monoclonal antibody to platelet glycoprotein IIb and IIIa, on this adhesion.

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Materials and Methods

Vitronectin was purified from normal plasma as described by Dahlbäck et al. with some modification (5, 31). Following barium citrate adsorption, PEG precipitation, DEAE Sephacel, Blue Sepharose and Sephacryl S200 chromatography the fractions containing vitronectin were applied to Concanavalin A Sepharose and vitronectin was eluted with α-methyl mannoside. The final preparation contained two bands at 75 kDa and 65 kDa when analyzed in SDS polyacrylamide gel electrophoresis (Fig. 1). Fibronectin was purified by affinity chromatography on gelatin-Sepharose as described (7). The murine monoclonal CP8, and IgG_1 directed against platelet glycoprotein IIb–IIIa complex, was a gift from Dr. Z. Ruggeri at Scripps Clinic and Research Foundation. This antibody is glycoprotein IIb-IIIa complex specific and does not cross react with other blood cells and endothelial cells. The monoclonal AP 1, an IgG_1 directed against platelet glycoprotein Ib and was gift from Dr. T. Kunicki of blood center of Wisconsin, Milwaukee. The control monoclonal antibody B66.7, an anti-Pan T cell, was a gift of Dr. B. Perussia and Dr. G. Trinchieri of Wistar Institute, Philadelphia, PA.

GRGDSP and GRGESP peptides were synthesized by Dr. Shan-Ho Chou of the Howard Hughes Medical Institute at the University of Washington. These peptides were over 95% homogeneous when analyzed in reverse phase HPLC (C-18 column). Human thrombin was a gift of Dr. John Fenton of State Dept of Health, Albany, NY. Standard laboratory chemicals were purchased from Sigma Chemical Company, St. Louis, MO. $Na_2{}^{51}CrO_4$ was obtained from New England Nuclear, Billerica, MA.

Isolation of Platelets

Blood was drawn through 19-gauge needles into polypropylene syringes containing to 1:9 volume of 3.8% sodium citrate pH 6.5. The blood was immediately transferred to polypropylene tubes and platelet rich plasma was obtained by centrifugation at 1,000 × g for 3 minutes at room temperature. Prostaglandin E_1 was added to the platelet rich plasma to a final concentration of 1 μM. After 10 minutes incubation platelets were sedimented by centrifugation at 1,000 × g for 12 minutes at room temperature. Following the initial spin platelets were suspended in 1 ml of platelet washing buffer (1) and incubated for 30 minutes with 100 Ci $Na^{51}CrO_4$. The platelet suspension was then washed thrice as described by Baenziger and Majerus (1). The platelets were suspended for adhesion assays in a pH 7.5 buffer containing 0.14 M NaCl, 0.012 M $NaHCO_3$, 0.00042 M Na_2HPO_4, 0.0027 M KCl, 5 mM HEPES, 5 mM dextrose and 10 mg/ml bovine serum albumin.

Preparation of Vitronectin Substrates

Substrates for platelet adhesion studies were prepared in 96 well ELISA plates (Falcon 3915, Becton, Dickinson and Co., Lincoln Park, NJ). A 200 μl solution containing various concentrations of vitronectin (0.005 μg–5 μg) in Tris-buffered saline was added to the wells and the plates were incubated at room temperature for 2 hours. After incubation, the solutions were aspirated and the wells were filled with buffer containing 1% (wt/vol) bovine serum albumin. Control wells were filled with BSA only. Fibronectin substrate wells were also prepared as positive controls in each experiment.

Adhesion Assays

Platelet adhesion assays were performed as described by Haverstick et al. (11). Following washing, platelets were suspended in the suspension

buffer at a concentration of ~2 × 10^8/ml. Calcium and magnesium chloride were added to a final concentration of 1 mM. Equal volumes of platelet suspension and antibodies or peptides were mixed and thrombin was added to a final concentration of 1 U/ml. The mixture was incubated for 20 minutes at room temperature without shaking. 100 µl aliquots of the platelet suspension were than transferred to the plates coated with the protein and incubated at room temperature for 30 minutes without shaking. At the end of the incubation period nonadherent platelets were removed by rinsing and aspiration in the binding buffer. The extent of binding was determined by incubating the wells sequentially with two 300 µl portions of 2% (wt/vol) of Sodium dodecyl sulfate for 20 minutes each. The extracts were pooled and the ^{15}Cr content was determined. In all experiments adhesion to fibronectin and albumin were performed as controls. As described by Haverstick et al. precautions are taken to avoid platelet aggregation during the incubation procedure, including avoidance of shaking and incubation at room temperature, rather than at 37° C (11).

Results

Adhesion of Platelets to Vitronectin Substrates

Platelets activated with thrombin adhere to polystyrene plates coated with vitronectin in a time dependent manner (Fig. 2). No significant binding was observed with unstimulated platelets to vitronectin or bovine serum albumin coated plates. Under these experimental conditions 25–35% of the added platelets adhere to the vitronectin substrate. Since this might represent a subpopulation of platelets, we collected the non-adherent platelets after 60 minutes and incubated them with fresh vitronectin coated wells. Again about 30–50% platelets adhere to the fresh substrate. In addition, the percentage of adherent platelets can be increased by performing the adhesion assay on a larger substrate surface such as a petri dish. Thus the plateau is due to the saturation of the

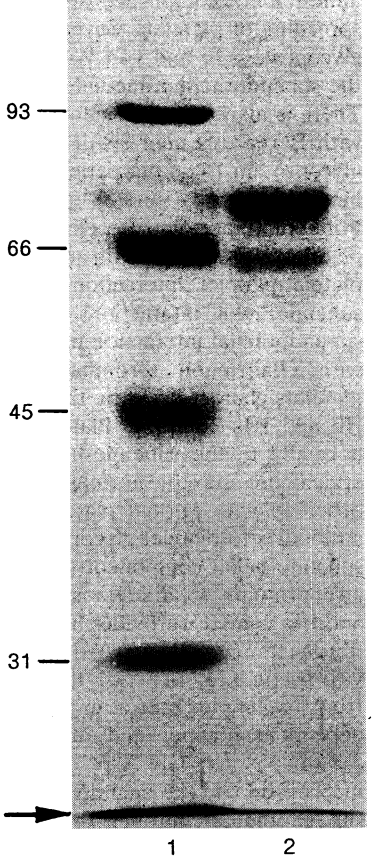

Fig. 1 Electrophorectic analysis of purified vitronectin. 10 µg of purified vitronectin was electrophoresed after reduction in 5% 2-mercaptomethanol, in 10% SDS-polyacrylamide gel. The gel was stained with Coomassie blue R250. The molecular weight standards are marked and the arrow indicates the position of the tracking dye

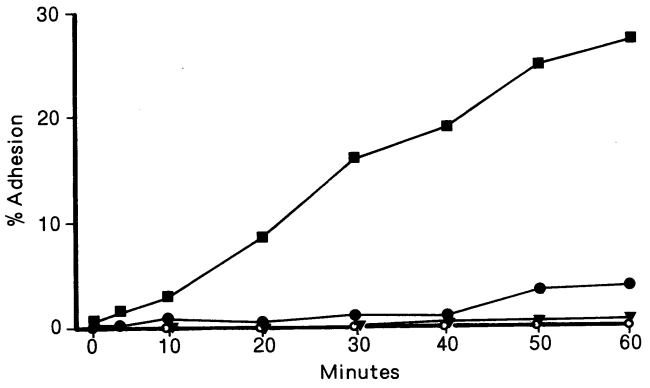

Fig. 2 Adhesion of platelets to vitronectin substrate: Platelets washed and labelled with $Na_2{}^{51}CrO_4$ and suspended in a buffer (0.15 M NaCl, 0.02 M Tris, 5 mM Dextrose and 1 mg/ml of bovine serum albumin). Platelets were activated with thrombin (1 U/ml) and transferred to polystyrene plates coated with vitronectin or albumin. The extent of adhesion is expressed as a percentage of platelets initially added to the plates. ■—■ Thrombin activated platelets on vitronectin; ▼—▼ control unactivated platelets on vitronectin; ●—● thrombin activated platelets on albumin; ○—○ control unactivated platelets on albumin

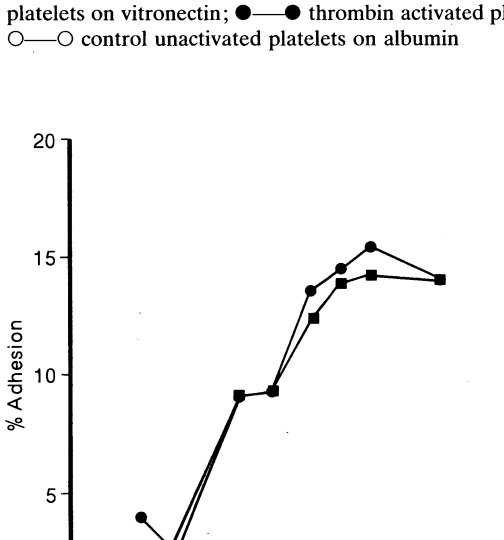

Fig. 3 Dose-dependent adhesion of thrombin activated platelets. The conditions were similar to Fig. 2, except the concentrations of vitronectin and fibronectin were changed. The extent of adhesion is expressed as a percentage of platelets initially added to the plates. ■—■ vitronectin substrate; ●—● fibronectin substrate; ▼—▼ albumin substrate

Table 1 Effect of divalent cations on thrombin-induced adhesion to vitronectin substrate

Assay conditions	Platelet adhesion (cpm bound)
No addition	513 ± 70
2 mM EDTA	47 ± 58
1 mM Ca	2224 ± 188
1 mM Mg	1404 ± 142
1 mM Mn	1339 ± 47

Washed, labelled and thrombin activated platelets were incubated with various cations or 2 mM EDTA and adhesion to vitronectin substrates was measured after 20 minutes. Results are the mean and one standard deviation of triplicate determinations.

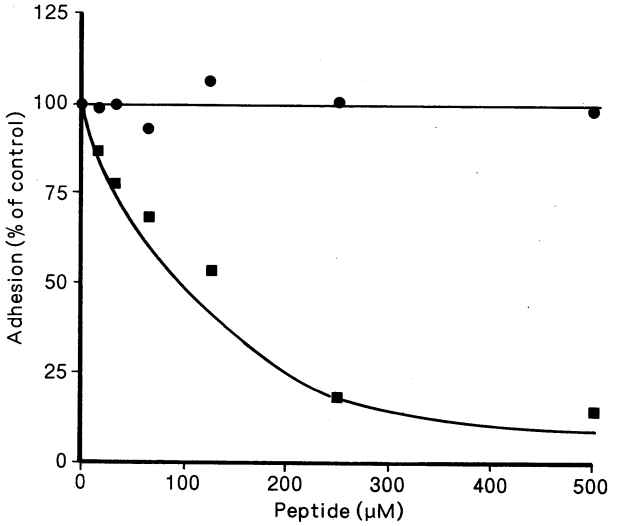

Fig. 4 Effect of synthetic peptides on platelet adhesion to vitronectin substrates. Platelets washed, labelled, activated with thrombin (1 U/ml) and incubated with various concentrations of the peptide for 20 minutes and then transferred to platelets coated with 1 μg/ml of vitronectin. Adhesion after 30 minutes was measured. ●—● GRGESP; ■—■ GRGDSP

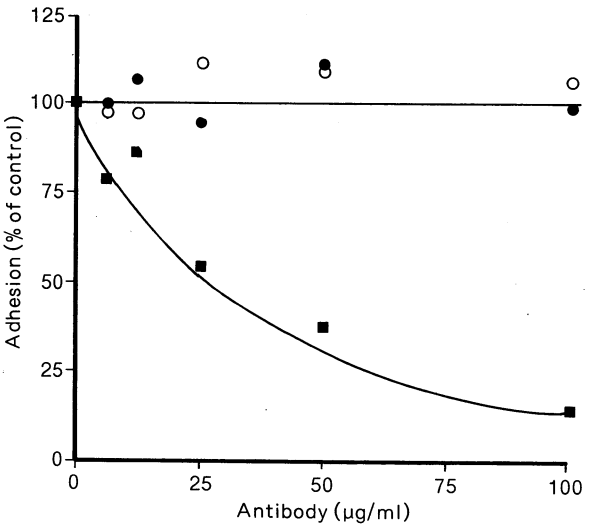

Fig. 5 Effect of monoclonal antibodies on platelet adhesion to vitronectin substrate. Platelets washed, labelled, activated with thrombin (1 U/ml) and incubated with various concentration of the monoclonal antibodies for 20 minutes before adhesion assay. Adhesion after 30 minutes was measured. ■—■, monoclonal antibody against glycoprotein IIb-IIIa complex; ○—○, monoclonal antibody against glycoprotein Ib; ●—●, isotype matched control

substrate with platelets. The adherent platelets, when viewed with the phase contrast microscope, were spread on the surface without significant aggregation. Thrombin stimulated platelets showed slight adhesion to albumin coated control plates compared to unactivated platelets which started after 40 minutes of incubation. At 20 minutes adhesion to vitronectin substrate represented 10–30% of total platelets added, while binding to albumin substrate was similar to unactivated platelets which were at most 2% or less. Adhesion also depends on the amount of vitronectin added to each wells (Fig. 3). Maximal adhesion occurred at 1 μg per well. For comparison, binding of platelets to fibronectin coated surfaces is also shown in Fig. 2.

Divalent Cation-Dependent Adhesion of Platelets to Vitronectin Substrates

The effect of various divalent cations on the adhesion of platelets to vitronectin substrates was determined in the presence or absence of thrombin. 2 mM EDTA almost completely inhibited platelet adhesion. Both manganese and magnesium supported platelet adhesion but less effective than calcium (Table 1).

Effect of RGD Containing Peptides on Platelet Adhesion to Vitronectin Substrates

The effect of RGD containing peptides on platelet adhesion to vitronectin coated surfaces was explored at a fixed concentration of vitronectin (1 μg per well). GRGDSP inhibited platelet adhesion to vitronectin substrate in a dose-dependent manner with half maximal inhibition occurring at a concentration of 80 μM (Fig. 4). The control peptide with the sequence GRGESP had no effect on platelet adhesion under identical conditions even up to 1 mM concentration.

Effect of Monoclonal Antibodies on Platelet Adhesion

The effect of a monoclonal antibody (CP 8) to platelet Gp IIb–IIIa complex on adhesion of platelets to vitronectin surfaces was examined. This antibody inhibited platelet adhesion to vitronectin in a dose-dependent manner, with half maximal inhibition at ~25 μg/ml (Fig. 5). An isotype matched control murine monoclonal antibody had no significant effect on platelet adhesion under the same conditions. In addition a monoclonal antibody to platelet glycoprotein Ib (AP 1) had no effect on this adhesion (Fig. 5).

Discussion

When endothelial continuity is disrupted platelets rapidly adhere to the subendothelial components that are exposed. This adhesion is accomplished by an initial attachment followed by the spreading of platelets. Several lines of evidence strongly suggest glycoprotein Ib and von Willebrand protein in plasma and/or in the subendothelium mediates initial attachment (27, 28, 32, 33). There is marked impairment of the initial attachment in patients with Bernard-Soulier syndrome whose platelets are deficient in glycoprotein Ib and in patients with von Willebrand disease who have quantitative and/or qualitative abnormalities of the von Willebrand protein. Recently it has been recognized that the platelet glycoprotein IIb and IIIa, in addition to their role in platelet-platelet interaction may also play a part in platelet-subendothelial interactions (28, 35). A major defect in platelet subendothelial interaction has been observed recently in patients with Glanzmann thrombasthenia, an inherited disorder of platelets characterized by the deficiency of platelet glycoprotein IIb and IIIa complex. Platelets in this disorder do not spread normally to the subendothelial matrix even though the initial attachment may be normal or increased. A similar defect was observed with normal platelets in the presence of monoclonal antibodies to platelet glycoprotein IIb and IIIa (28). The precise subendothelial component(s) mediating this interaction through glycoproteins IIb and IIIa is not known. Platelet glycoprotein IIb and IIIa complex provides binding sites for fibrinogen (4, 10, 16), fibronectin (8, 18), von Willebrand protein (24, 25) and also for vitronectin (23, 31). All these ligands react with platelet Gp IIb–IIIa complex only after platelet activation in a divalent cation dependent manner and the binding is inhibited by peptides containing Arg-Gly-Asp containing sequences. The precise mechanism and the ligand(s) involved in platelet adhesion to the subendothelium through the glycoproteins IIb and IIIa mecha-

nism remain to be elucidated. In this context it is interesting to note that platelet subendothelial reaction is markedly impaired in storage pool disorders suggesting released alpha granule components may be involved in this interaction (35). It has been shown that fibronectin in addition to its role in supporting platelet adhesion to collagenous subendothelial surface (14) is also required for thrombus formation (3). Fibrinogen is unlikely to be involved in this interaction since platelet subendothelial interaction is normal in a patient with afibrinogenemia (35).

The results of this study suggest that thrombin stimulated platelets adhere to vitronectin substrates in a manner similar to the adhesion previously described for fibrinogen, fibronectin and von Willebrand protein under static conditions (11). Under these experimental conditions there is no activation-independent adhesion unlike the adhesion shown for fibronectin (17). The physiological relevance of this interaction is not clear. These studies were performed under static conditions and as such may not be totally applicable to flowing blood. It has recently been shown, under flow conditions fibronectin-dependent platelet adhesion is not dependent upon RGD(S) mechanism (17), while under static condition, the adhesion to fibronectin substrate is dependent upon RGD(S) mechanism (11). Further studies should be performed in flowing blood to to delineate the role of vitronectin in platelet-vessel wall interaction. Vitronectin localizes in the extracellular matrix (2, 6) and in addition interacts with native collagen (9) and thus may provide a substrate for platelet adhesion and spreading. It also neutralizes heparin inactivation of thrombin and factor Xa by antithrombin III (19, 20, 22). Vitronectin may play a role in the platelet-vessel wall interactions by providing a substrate for platelets and by modulating thrombin inactivation by antithrombin III.

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