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Interaction of Thrombin-activated Platelets with Extracellular Matrices (Fibronectin and Vitronectin): Comparison of the Activity of Arg-Gly-Asp-containing Venom Peptides and Monoclonal Antibodies Against Glycoprotein IIb/IIIa Complex

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Abstract

Platelets adhere to fibronectin and vitronectin substrates following activation with physiological concentrations of thrombin. Adhesion of activated-platelets to either substrate is dependent upon the amount of fibronectin and vitronectin, and the duration of the adhesion assay. In this study, we showed that the Arg-Gly-Asp-containing peptides (including naturally occurring polypeptides, triflavin, trigramin and rhodostomin, synthetic peptides GRGDS, GRGDSPK, GRGDF, and GRGD and monoclonal antibodies, 7E3, 10E5 and AP2, raised against glycoprotein IIb/IIIa complex, inhibited the adhesion of activated-platelets to fibronectin and vitronectin-coated plates in a dose-dependent manner.

In fibronectin-coated plates, GRGDF was shown to be much more efficient than GRGDS, GRGDSPK and GRGD at inhibiting the adhesion of activated-platelets to immobilized fibronectin. On the other hand, there were no marked differences in the abilities of these three peptides (GRGDF, GRGDS and GRGDSPK) to inhibit platelet adhesion to immobilized vitronectin. Furthermore, the RGD-containing venom peptide, triflavin was more effective than rhodostomin and trigramin at inhibiting the adhesion of activated-platelets to either substrates. The monoclonal antibodies raised against glycoprotein IIb/IIIa complex (i.e., 7E3, 10E5 and AP2) inhibited platelet adhesion to fibronectin and vitronectin in a similar dose-dependent manner. Interestingly, we found that 7E3 was more efficient than 10E5 and AP2 in this reaction.

These studies suggest that the glycoprotein IIb/IIIa complex, present on activated-platelets, may interact with fibronectin and vitronectin substrates through the Arg-Gly-Asp-dependent mechanism. Since fibronectin and vitronectin are present in the subendothelial matrix, they may be involved in platelet-vessel wall interaction. The Arg-Gly-Asp containing peptide, especially triflavin, is an ideal therapeutic agent for inhibiting thrombus formation by interrupting platelet-platelet and platelet-subendothelium interactions.

When the endothelial cell layer is disrupted, platelets accumulate on the exposed subendothelium. Platelet attachment, spreading and aggregation on vascular subendothelium are crucial events in platelet thrombus formation. Several factors are known to regulate these events: physical factors (Turitto & Baumgartner 1979; Arats et al 1983), subendothelial components (Sixma 1981), adhesive proteins (Houdijk et al 1986) and specific receptors on the platelet membrane (Coller et al 1991).

Fibronectin is a disulphide-linked glycoprotein which is synthesized by a variety of cells and is found on cell surfaces, in the extracellular matrix, in plasma and in other body fluids (Hynes & Yamada 1982). Each of its similar, but not necessarily identical, peptide chains is composed of a series of relatively protease-resistant domains which contain binding sites for macromolecules such as collagen, glycosaminoglycans, fibrin, and a cell surface receptor that mediates the adhesion and spreading of cells on fibronectin-containing substrates. Studies have revealed that fibronectin is located within the α granules of platelets and is secreted upon platelet activation (Zucker et al 1979). Fibronectin is not expressed on the surface of unactivated platelets, but does become platelet surface-associated following activation (Ginsberg et al 1980). This association is presumably mediated by the expression of specific fibronectin receptors on platelets following activation (Plow & Ginsberg 1981). Furthermore, there is much evidence that the Arg-Gly-Asp sequence within fibronectin is involved in the cell attachment function of this glycoprotein (Haverstick et al 1985).

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 (Pytela et al 1986). Using monoclonal antibodies, it has been shown that vitronectin is associated with platelets among blood cells (Barnes & Silnutzer 1983). Liposomes containing platelet glycoproteins IIb and IIIa are bound to vitronectin substrates by the Arg-Gly-Asp-dependent mechanism (Pytela et al 1986).

Recently, many trigramin-like antiplatelet peptides (termed 'disintegrin') have been described (Gould et al 1990; Huang et al 1991a,b). Trigramin, a single chain (MW $\sim$7500), cysteine-rich peptide purified from the venom of the *Trimeresurus gramineus* snake venom contains the Arg-Gly-Asp sequence and is a highly specific fibrinogen receptor antagonist with binding affinity (K_d, 20 nM) for the activated platelet fibrinogen receptor (Huang et al 1987;, 1989). These peptides all containing Arg-Gly-Asp, are rich in cysteine, and bind with high affinity to integrin on the surface of platelets and other cells (Knudsen et al 1988; Gould et al 1990; Sheu et al 1992a,b,c, 1993, 1994b,c). Triflavin, a trigramin-like antiplatelet peptide purified from *Trimeresurus flavoviridis* snake

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venom (Huang et al 1991c,d) is more potent than trigramin. Its primary structure consists of 70 amino acid residues including 12 cysteines with the Arg-Gly-Asp sequence at position 49–51 (Huang et al 1991e). Triflavin inhibits human platelet aggregation stimulated by thrombin, collagen, and U46619, not only in washed human platelets but also in platelet-rich plasma and whole blood (Huang et al 1991c). We previously reported that triflavin inhibits platelet aggregation by interfering with the interaction of fibrinogen with its specific receptor associated with the glycoprotein IIb/IIIa complex (Huang et al 1991e; Sheu et al 1992c). It is now well established that the binding of fibrinogen to its receptor associated with a Ca^2-dependent glycoprotein IIb/IIIa complex ($\alpha_{IIb}\beta_3$ integrin) is the common mechanism of platelet aggregation stimulated by agonists (Phillips et al 1988). The platelet membrane glycoprotein IIb/IIIa complex, a member of the family of Arg-Gly-Asp (RGD)-binding adhesive protein receptors serves as an activation-dependent receptor for plasma adhesive protein, i.e. fibrinogen, fibronectin, and von Willebrand factor (vWF) (Pytela et al 1986). Therefore, peptides containing the Arg-Gly-Asp sequence may partially or fully inhibit fibrinogen binding to its specific receptor associated with the glycoprotein IIb/IIIa complex (Kloczewiak et al 1984; Ginsberg et al 1985). On the other hand, monoclonal antibodies directed against the glycoprotein IIb/IIIa complex have been demonstrated to be potent inhibitors of platelet aggregation in-vivo (Coller et al 1991).

In this study, we have examined whether surface bound fibronectin and vitronectin can serve as efficient substrates for platelet adhesion at resting and also following stimulation. The structure-function relationships required for platelet-substrate interaction were explored by the use of a series of Arg-Gly-Asp-related synthetic peptides or naturally occurring venom peptides. Furthermore, we also compared the activity of peptides containing the Arg-Gly-Asp sequence (i.e., disintegrin and synthetic peptides) with those of monoclonal antibodies directed against the glycoprotein IIb/IIIa complex on this adhesion.

Materials and Methods

Materials

Trimeresurus flavoviridis venom, *T. gramineus* venom and *Agkistrodon rhodostoma* (or *Calloselasma rhodostoma*) venom were purchased from Latoxan (France), and stored at −20°C. Triflavin, trigramin and rhodostomin were purified from the venom of *T. flavoviridis, T. gramineus* and *A. rhodostoma*, respectively, as previously described (Huang et al 1989, 1990, 1991c). Gly-Arg-Gly-Asp-Ser (GRGDS), Arg-Gly-Asp-Ser (RGDS), and Gly-Arg-Gly-Asp-Ser-Pro-Lys (GRGDSPK) were purchased from Peninsula Laboratories, Calif., USA. Gly-Arg-Gly-Glu-Ser (GRGES) was synthesized by the Biochemical Institute, College of Medicine, National Taiwan University. Gly-Arg-Gly-Asp-Phe (GRGDF) was synthesized by the Tekon Scientific Corp. Apyrase (grade III), heparin, thrombin (from human plasma), prostaglandin E_1 (PGE_1), fibronectin (from bovine plasma) and vitronectin (from human plasma) were obtained from Sigma (St. Louis, MO, USA). Monoclonal antibodies 7E3 and 10E5 raised against the platelet membrane glycoprotein IIb/IIIa complex were kindly supplied by Dr. B. Coller (State University of New York, Stony Brook, NY, USA). AP_1 raised against the platelet membrane glycoprotein Ib, AP_2 raised against the glycoprotein IIb/IIIa complex and AP_3 raised against the glycoprotein IIIa were kindly donated by Drs P. Newman and T. Kuniki (Milwaukee Blood Center, Milwaukee, Wis, USA). Monoclonal antibodies MCA 757 (anti-$\alpha_v\beta_3$), MCA 698 (anti-$\alpha_5\beta_1$) and MCA699 (anti-$\alpha_6\beta_1$) were purchased from Serotec Ltd. (Bicester, UK). 2′-7′-Bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein acetoxy-methyl (BCECF-AM) was purchased from Molecular Probes, Inc. (Eugene, OR, USA).

Preparation of BCECF/AM-labelled human washed platelet suspension

Blood was collected from healthy human volunteers, who had not taken any drugs within the preceding 2 weeks. Blood was mixed with acid citrate dextrose (9:1, v/v). Following centrifugation for 10 min at 120 g at room temperature, the supernatant (platelet-rich plasma) was supplemented with PGE_1 (0·5 μM) and heparin (6·4 units mL^{-1}), incubated for 10 min at 37°C and centrifuged at 500 *g* for 10 min. The platelet pellet was suspended in 5 mL of Ca^2-free Tyrode's solution (pH 7·3), and then apyrase (1 unit mL^{-1}), PGE_1 (0·5 μM), heparin (6·4 Units mL^{-1}) and BCECF/AM (5 μM) were added, followed by incubation for 40 min at 37°C. Following centrifugation of the suspension at 500 *g* for 6 min, the washing procedure was repeated. The washed platelets were finally suspended in Ca^2-free Tyrode's solution (3×10^8 platelets mL^{-1}), containing 1 μM of PGE_1.

Preparation of matrix coating

Substrates for platelet adhesion studies were prepared in plates (96-well; Costar, USA). Fifty microlitres of various concentrations of fibronectin and vitronectin (0·05 ~ 1·5 μg per well) in phosphate-buffered saline was added to the wells and the plates were incubated at room temperature for 4 h. After incubation, the solutions were aspirated and the wells were filled with buffer containing bovine serum albumin (0·5%). Control wells were filled with BSA only.

Adhesion assays

Platelet adhesion assays were performed as described by Haverstick et al (1985). Equal volumes of platelet suspension and antibodies or peptides were mixed and thrombin was added to a final concentration of 0·1 units mL^{-1}. The mixture was incubated for 20 min at room temperature without shaking. Fifty-microlitre aliquots of platelet suspension (3×10^8 platelets mL^{-1}) were then transferred to the well coated with the matrices, and incubated at room temperature for 30 min without shaking. Nonadherent platelets were removed by aspiration and the platelets were gently washed three times with Ca^2-free Tyrode's solution. The extent of binding was determined with a CytoFluor 2300 fluorescence plate reader (Millipore, Bedford, MA, USA).

Statistical analysis

The experimental results are expressed as the mean ± s.e.m. and accompanied by the number of observations. Data was assessed by the method of analysis of variance. If this analysis indicated significant differences among the group means, then each group was compared by the Newman-Keuls method. A P value of less than 0·05 was considered significant.

Results

Adhesion of platelets to fibronectin and vitronectin substrates

2',7'-Bis(2-carboxyethyl)-5 (and-6)-carboxyfluorescein acetoxy-methyl (BCECF-AM) has been used in fluorescence-based viability assessment in adherent cell cultures (Vaporciyan et al 1993). In this study, we evaluated the extent of thrombin-activated platelet adhesion to plates coated with fibronectin and vitronectin substrates by using BCECF-labelled platelets instead of conventional isotope-labelled platelets. Fig. 1 demonstrates that thrombin-activated platelets adhere to fibronectin- and vitronectin-coated plates in a time-dependent manner. No significant binding was observed with unactivated platelets to fibronectin- and vitronectin-coated wells. Under these experimental conditions 20–30% of the added platelets adhered to the fibronectin and vitronectin substrates. Since this might represent a subpopulation of platelets, we collected the non-adherent activated platelets after 60 min and incubated them with fresh fibronectin- and vitronectin-coated wells. Again about 25–35% activated-platelets adhered to the fresh substrate. In addition, the percentage of the adhering platelets could be significantly increased by performing the adhesion assay on a larger substrate surface such as a 48-well plate. Therefore, the plateau is due to the saturation of the substrates with platelets. Furthermore, the adhering platelets, when viewed under a phase contrast microscope, were found to be spread on the surface without significant aggregation. For the same concentration (0·5 μg per well), the maximal adhesion of activated-platelets to fibronectin and vitronectin, following the addition of platelets to wells, was reach at about 30 and 40 min, respectively (Fig. 1). Therefore, in subsequent experiments, we used an appropriate incubation time (40 min) to characterize the inhibitory effect of RGD-containing peptides or monoclonal antibodies on adhesion assays. Thrombin-activated platelets showed no significant adhesion to bovine serum albumin (BSA)-coated control plates which started after 40 min of incubation (Fig. 2), indicating that the percent adhesion of activated-platelets to BSA was similar to the percent adhesion of unactivated-platelets to fibronectin- and vitronectin-coated plates, being 5% or less (Figs 1, 2).

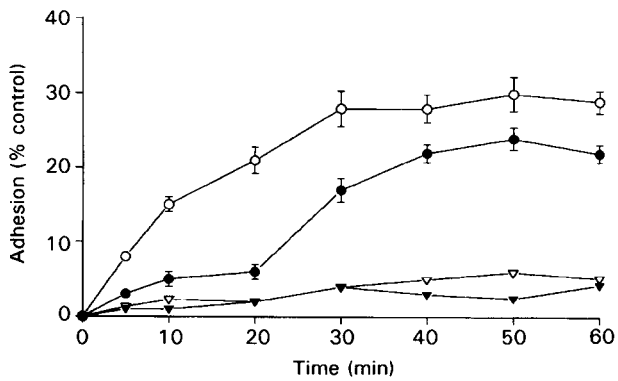

FIG. 1. Adhesion of platelets to fibronectin and vitronectin substrates. Platelets were activated with thrombin (0·1 units mL^{-1}) and transferred to plates coated with fibronectin and vitronectin. The extent of adhesion is expressed as a percentage of platelets initially added to the plates. Thrombin-activated platelets on fibronectin (○); thrombin-activated platelets on vitronectin (●); unactivated platelets on fibronectin (▽); unactivated platelets on vitronectin (▼). Data are presented as mean ± s.e.m. (n = 5).

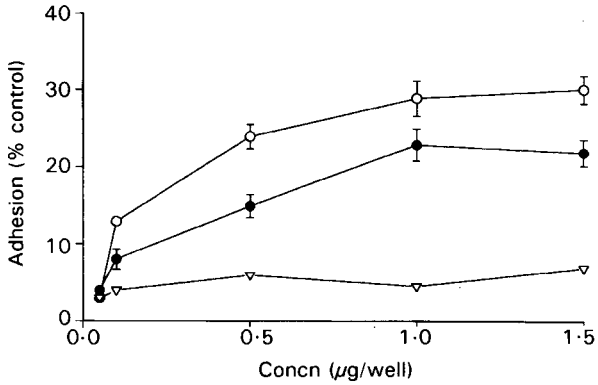

FIG. 2. Dose-dependent adhesion of thrombin-activated platelets. The experimental conditions were similar to those for Fig.1 except that the concentrations of fibronectin and vitronectin were changed. The exent of adhesion is expressed as a percentage of platelets initially added to the plates. Fibronectin (○); vitronectin (●); bovine serum albumin (▽). Data are presented as mean ± s.e.m. (n = 5).

On the other hand, adhesion also depends on the amount of fibronectin and vitronectin added to each well (Fig. 2). The maximum adhesion with fibronectin (about 26%) and vitronectin about 20%) occurred at concentrations of 0·5 μg per well and 1·0 μg per well, respectively. These results indicate that the activated-platelets adhered more efficiently to fibronectin than vitronectin. In subsequent experiments, we also used appropriate concentration of fibronectin (0·5 μg per well) and vitronectin (1·0 μg per well) in adhesion assays.

Effects of RGD-containing synthetic peptides on platelet adhesion to fibronectin and vitronectin substrates

The effect of RGD-containing peptides on platelet adhesion to fibronectin- and vitronectin-coated surfaces was explored at fixed concentrations of fibronectin (0·5 μg per well) and vitronectin (1·0 μg per well). The relative effectiveness of the synthetic peptides was more carefully examined by testing the adhesion of platelets to fibronectin and vitronectin over a wide range of peptide concentrations. The results are shown in Fig. 3. At 0·5 mM, GRGDF almost completely inhibited platelet attachment (85%) (Fig. 3). There were no marked differences ($P < 0.05$) in the abilities of the two peptides containing the RGDS sequence (GRGDS and GRGDSPK) to inhibit the adhesion of activated-platelets to fibronectin-coated plates when they were compared on a molar basis. As shown in Fig. 3A, the reduced effectiveness of the peptide GRGD is very apparent ($P < 0.05$ as compared with GRGDS). The concentration of this peptide required to achieve 50% inhibition of adhesion (IC50, 0·77 mM) was nearly two orders of magnitude greater than that required for a similar extent of inhibition by the peptide GRGDF (IC50, 0·31 mM). Therefore, GRGDF was much more efficient than GRGDS, GRGDSPK and GRGD at blocking the adhesion of activated-platelets to fibronectin substrate (Fig. 3A). On the other hand, there were no marked differences in the abilities of the three peptides (GRGDF, GRGDS and GRGDSPK) to inhibit the adhesion of activated-platelets to vitronectin substrate ($P < 0.05$) (Fig. 3B), however, GRGD was less effective in blocking platelet adhesion to vitronectin ($P < 0.05$ as compared with GRGDS) (Fig. 3B). In contrast, GRGES (1 mM) had no significant effect on platelet adhesion to either substrate under identical conditions (Fig. 3),

suggesting that RGD-containing peptides may specifically interrupt the adhesion of activated-platelets to immobilized fibronectin and vitronectin.

Effect of RGD-containing venom peptides on the adhesion of activated platelets to immobilized fibronectin and vitronectin

Inhibition of the adhesion of activated-platelets to fibronectin and vitronectin by various concentrations of RGD-containing venom peptides is shown in Fig. 4. Adhesion of platelets to fibronectin and vitronectin was inhibited by triflavin, rhodostomin and trigramin in a dose-dependent manner. At 6 μM, triflavin almost completely inhibited platelet adhesion to fibronectin (95%). At the same concentration (6 μM), triflavin inhibited platelet adhesion to vitronectin by only about 73%. There were no obvious differences ($P > 0.05$) in the abilities of rhodostomin and trigramin to inhibit the adhesion of activated-platelets to either substrate (Fig. 4). The results also indicated that triflavin was also more efficient than rhodostomin and trigramin at inhibiting the adhesion of activated-platelets to either substrates ($P < 0.05$ as compared with trigramin).

Effect of monoclonal antibodies on platelet adhesion

The effect of monoclonal antibodies raised against platelet glycoprotein (GP) IIb/IIIa complex (i.e., 7E3, 10E5 and AP_2), GP IIIa (AP_3) and GP Ib (AP_1) on the adhesion of activated-platelets to fibronectin and vitronectin substrates was examined. The monoclonal antibodies raised against GP IIb/IIIa complex (i.e., 7E3,10E5 and AP_2) inhibited platelet adhesion to fibronectin and vitronectin in a similar dose-dependent manner. Interestingly, 7E3 was shown to be more efficient than 10E5 and AP_2 at inhibiting the adhesion of platelets to either substrate ($P < 0.05$) (Fig. 5). On the other hand, AP_3 was shown to only slightly inhibit platelet adhesion to either substrate ($P < 0.05$ as compared with AP_1). AP_1 had no effect on this adhesion (Fig. 5). We hypothesized that other integrin receptors may mediate activated-platelets adhesion to immobilized fibronectin and vitronectin. Therefore, we further

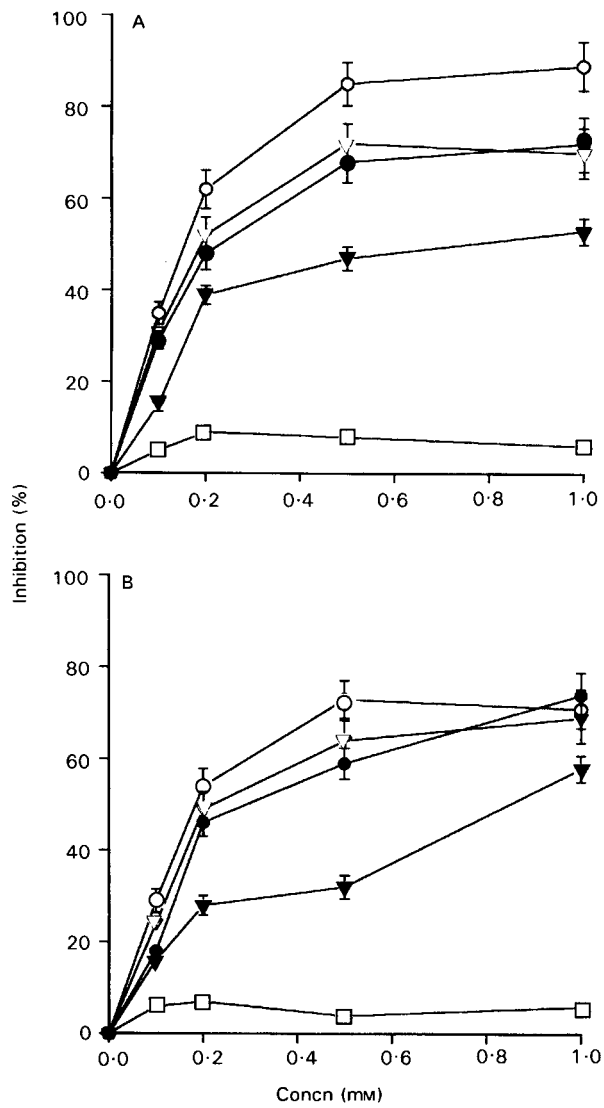

FIG. 3. Effect of synthetic peptides on thrombin-activated platelet adhesion to (A) fibronectin and (B) vitronectin substrates. Platelets were washed, labelled, activated with thrombin (0·1 units mL^{-1}) and incubated with various concentrations of the peptide for 20 min and then transferred to plates coated with fibronectin (0·5 μg per well) and vitronectin (1·0 μg per well). Adhesion after 40 min was measured. GRGDF (○); GRGDS (●); GRGDSPK (▽); GRGD (▼); GRGES (□). The extent of adhesion is expressed as a percentage of platelets initially added to the plates. Data are presented as mean ± s.e.m. (n = 5).

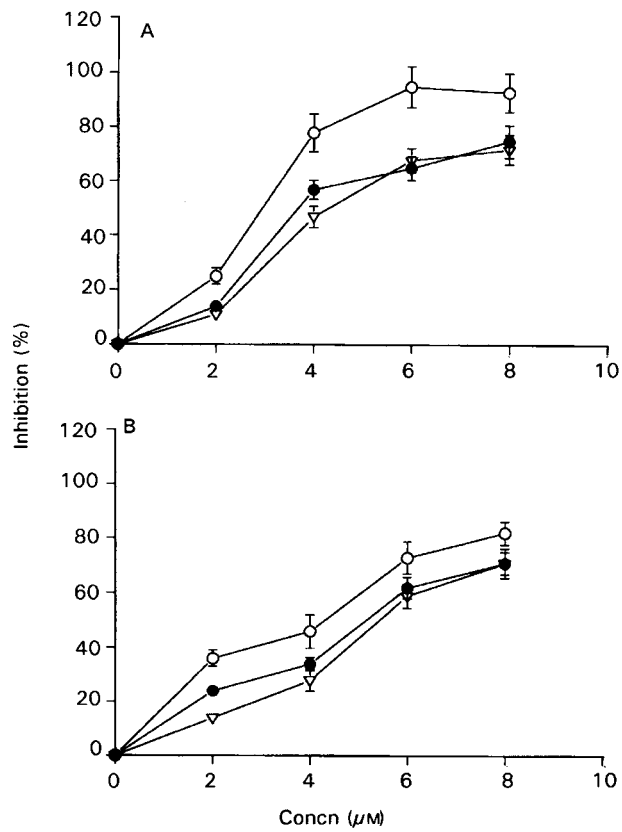

FIG. 4. Effect of Arg-Gly-Asp-containing venom peptides on thrombin-activated platelet adhesion to (A) fibronectin and (B) vitronectin substrates. The experimental conditions were similar to those for Fig. 3. Triflavin (○); rhodostomin (●); trigramin (▽). Data are presented as mean ± s.e.m. (n = 5).

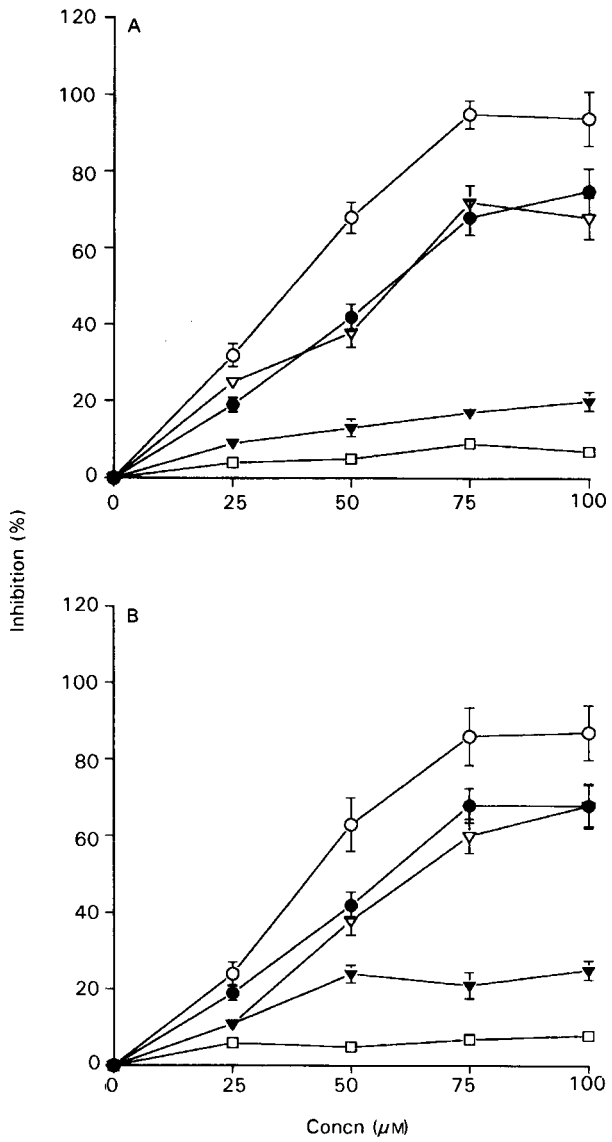

FIG. 5. Effect of monoclonal antibodies on thrombin-activated platelet adhesion to (A) fibronectin and (B) vitronectin. The experimental conditions were similar to those for Fig. 3. 7E3 (○); 10E5 (●); AP_2 (▽); AP_3 (▼); AP_1 (□). Data are presented as mean ± s.e.m. (n = 5).

characterized this reaction by using monoclonal antibodies against other integrin receptors on platelet membrane as follows: MCA698 (anti-$\alpha_5\beta_1$), MAC699 (anti-$\alpha_6\beta_1$) and MCA757 (anti-$\alpha_v\beta_3$). As shown in Table 1, MCA 698, 757 and 699 had no significant effect on this adhesion.

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 spreading of platelets. Evidence strongly suggests that platelet GP Ib and von Willebrand factor in plasma, subendothelium, or both, mediate initial attachment (Turitto et al 1985; Sakariassen et al 1986). There is marked impairment of the initial attachment in patients with Bernard-Soulier syndrome whose

Table 1. Effect of anti-$\alpha_{IIb}\beta_3$ (7E3), anti-$\alpha_5\beta_1$ (MCA 698), anti-$\alpha_v\beta_3$ (MCA 757) and anti-$\alpha_6\beta_1$ (MCA 996) monoclonal antibodies (1:200 dilutions of stock) on binding of thrombin-activated platelets to fibronectin or vitronectin substrates.

	Inhibition (%) of adherence to	
	Fibronectin	Vitronectin
7E3	94 ± 7	87 ± 7·1
MCA698	14 ± 2·8	9 ± 3
MCA757	12 ± 1·9	15 ± 2·9
MCA699	9 ± 1·4	12 ± 1·9

The experimental conditions were similar to those in Fig. 3. Data are presented as mean ± s.e.m. (n = 5).

platelets are deficient in GP Ib and in patients with von Willebrand disease who have quantitative or qualitative abnormalities, or both, of the von Willebrand protein. Several studies have also demonstrated that the platelet GP IIb/IIIa complex also plays a part in platelet-subendothelial interactions (Sakariassen et al 1986; Weiss et al 1986). A major defect in platelet subendothelial interaction has been observed in patients with Glanzmann thrombasthenia, an inherited disorder of platelet GP IIb/IIIa complex. Platelets in patients with this disorder do not spread normally to the subendothelial matrix even though the initial attachment may be normal or increased. Platelet GP IIb/IIIa complex provides binding sites for fibrinogen (Gogstad et al 1982; Nachman & Leung 1982), fibronectin (Gardner & Hynes 1985), von Willebrand factor (Ruggeri et al 1983) and also for vitronectin (Pytela et al 1986). 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 sequences. The precise mechanism and the ligand(s) involved in platelet adhesion to the subendothelium through the GP IIb/IIIa complex mechanism, remain to be elucidated. It has been shown that fibronectin, in addition to its role in supporting platelet adhesion to the collagenous subendothelial surface (Houdijk & Sixma 1985), is also required for thrombus formation (Bastida et al 1987). Fibrinogen is unlikely to be involved in this interaction since platelet subendothelial interaction was normal in a patient with afibrinogenaemia (Weiss et al 1986).

The results of this study suggest that thrombin-activated platelets adhere to fibronectin and vitronectin substrates in a dose-dependent manner similar to the adhesion previously described for fibrinogen and von Willebrand factor under static conditions (Haverstick et al 1985). It has recently been shown, under flow conditions that fibronectin-dependent platelet adhesion is not dependent upon the RGD(S) mechanism (Nievelstein et al 1988), while under static conditions, the adhesion to fibronectin substrate is dependent upon the RGD(S) mechanism (Haverstick et al 1985). Vitronectin localizes in the extracellular matrix (Barnes & Silnutzer 1983) and in addition interacts with native collagen (Gebb et al 1986) and thus may provide a substrate for platelet adhesion and spreading. It also neutralizes heparin inactivation of thrombin and factor Xa by antithrombin III (Preissner & Muller-Bergaus 1987). Vitronectin may play a role in platelet-vessel well interactions by providing a substrate for platelets.

The series of experiments described in this report were performed to examine whether or not peptides containing the RGD sequence can effectively inhibit the adhesion of thrombin-activated platelets to fibronectin and vitronectin substrates. Alterations of the sequence of amino acid residues within these small peptides by transposition of amino acids significantly changed their activity (Fig. 3). The peptide GRGD, in which the carboxyterminal serine was deleted, retained some activity, although a nearly 2-fold higher concentration of this peptide was required to inhibit, by 50%, the adhesion of activated-platelets to either substrate. Platelets, therefore, appear to recognize the same small peptide of fibronectin and vitronectin. These were, however, interesting differences observed in that GRGDF was significantly more efficient than GRGDS or GRGDSPK in fibronectin-coated plates, whereas, the activity of the three peptides did not differ significantly in vitronectin-coated plates. It has been reported that the synthetic peptide RGDF is 4- to 5-fold more efficient than RGDS in inhibiting fibrinogen binding (Plow et al 1987). These results may explain why GRGDF was more efficient than GRGDS in fibronectin-coated plates. This finding has also been supported by studies in which triflavin (containing RGDF) was found to be significantly more efficient than rhodostomin (containing RGDM) and trigramin (containing RGDD) in fibronectin- and vitronectin-coated substrates (Fig. 4). We have previously described that triflavin was 3-fold more efficient than trigramin at inhibiting platelet aggregation in vitro (Sheu et al 1994c), and ex-vivo (Sheu & Huang 1994). Recently, we further reported that triflavin was more efficient than trigramin in preventing thrombosis in-vivo models (Sheu et al 1994a, 1995).

In the case of anti-GP IIb/IIIa, anti-GP IIIa or anti-GP Ib monoclonal antibodies, it was interesting to find that 7E3 was significantly more efficient than the other monoclonal antibodies raised against GP IIb/IIIa (i.e., AP_2 and 10E5) (Fig. 5). In previous studies, GPIIIa (β_3 subunit) was selectively cross-linked to fibrinogen and to the 12 KDa fragment of fibronectin (Bennett et al 1982; Gardner & Hynes 1985). Furthermore, vitronectin receptor ($\alpha_v\beta_3$) also shares the same β_3 subunit on the platelet membrane. Taken together, these results suggest that a site on or proximal to GPIIIa serves as a component of the functional adhesive receptor on platelets (i.e., fibrinogen, fibronectin or vitronectin) (Kornecki et al 1984; Sheu et al 1992c). Grossi et al. (1989) have reported that monoclonal antibody 10E5 is directed against an epitope on the GPIIb/IIIa complex and does not precipitate monomeric dissociated glycoprotein. In contrast, monoclonal antibody 7E3 appears to recognize an antigenic determinant on the β-subunit of GPIIb/IIIa (i.e., GPIIIa) (Sheu et al 1992c). In addition, we previously reported that 7E3 shows a profound inhibition of [^{125}I]triflavin binding to platelets whereas 10E5 and AP2 show only a slight inhibition at the same concentration (Huang et al 1991e). Therefore, it may be that 7E3 and triflavin have a similar binding epitope on, or proximal to, GP IIIa on the platelet membrane, resulting in 7E3 or triflavin being more efficiently activity at inhibiting the adhesion of activated-platelets to immobilized fibronectin and vitronectin. This may explain why 7E3 was more efficient than 10E5 and AP_2 or triflavin was more efficient than rhodostomin and trigramin at inhibiting the adhesion of activated-platelets to fibronectin and vitronectin substrates (Fig. 5).

In addition to the major fibrinogen receptor on platelets, GP IIb/IIIa ($\alpha_{IIb}\beta_3$), is present in large numbers ($\sim$40 000 receptors per platelet). Platelets (and presumably megakaryocytes) also contain several other integrins, including vitronectin ($\alpha_v\beta_3$), collagen ($\alpha_2\beta_1$), fibronectin ($\alpha_5\beta_1$), and laminin ($\alpha_6\beta_1$) receptors. One of these, $\alpha_v\beta_3$, also binds fibrinogen, fibronectin and von Willebrand factor (Charo et al 1987), but is present in very low numbers ($\sim$50–100 per platelet) (Coller et al 1991). It is necessary to further characterize the adhesive properties of activated platelets, and to investigate whether or not there are other integrins expressed on the platelet membrane which may mediate platelet adhesion to immobilized fibronectin and vitronectin. However, monoclonal antibodies anti-$\alpha_5\beta_1$, -$\alpha_v\beta_3$ and -$\alpha_6\beta_1$ had no significant inhibiting effect on the adhesion of activated-platelets to fibronectin and vitronectin, indicating that $\alpha_5\beta_1$ (fibronectin receptor), $\alpha_v\beta_3$ (vitronectin receptor) and $\alpha_6\beta_1$ (laminin receptor) do not mediate platelet adhesion to immobilized fibronectin and vitronectin (Table 1).

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