Interaction of disintegrins with the $\alpha_{IIb}\beta_3$ receptor on resting and activated human platelets

Mary Ann McLANE,* Maria Anna KOWALSKA,* Lee SILVER,* Sanford J. SHATTIL† and Stefan NIEWIAROWSKI*‡

*Department of Physiology and Sol Sherry Center for Thrombosis Research, Temple University School of Medicine, Philadelphia, PA 19140, U.S.A. and †Hematology–Oncology Division, Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, U.S.A.

Viper venom disintegrins contain the RGD/KGD motif. They inhibit platelet aggregation and cell adhesion, but show structural and functional heterogeneity. We investigated the interaction of four prototypic disintegrins with $\alpha_{IIb}\beta_3$ expressed on the surface of resting and activated platelets. The binding affinity (K_d) of ^{125}I-albolabrin, ^{125}I-echistatin, ^{125}I-bitistatin and ^{125}I-eristostatin toward resting platelets was 294, 153, 48 and 18 nM respectively. The K_d value for albolabrin decreased 3-fold and 6-fold after ADP- or thrombin-induced activation. The K_d values for bitistatin and echistatin also decreased with ADP, but there was no further decrease with thrombin. In contrast, eristostatin bound with the same high affinity to resting and activated platelets. The pattern of fluorescein isothiocyanate (FITC)–eristostatin and FITC–albolabrin binding to resting and activated platelets was consistent with observations using radiolabelled material. Eristostatin showed faster and more irreversible binding to platelets, and greater potency compared with albolabrin in inducing conformational neo-epitopes in β_3. The anti-$\alpha_{IIb}\beta_3$ monoclonal antibody OP-G2 that is RGD-dependent inhibited disintegrin binding to activated platelets more strongly than binding to resting platelets and it inhibited the binding to platelets of albolabrin more strongly than eristostatin. The specificity of disintegrin interaction with $\alpha_{IIb}\beta_3$ was confirmed by demonstrating cross-linking of these peptides to $\alpha_{IIb}\beta_3$ on normal platelets, but not to thrombasthenic platelets deficient in $\alpha_{IIb}\beta_3$.

INTRODUCTION

Platelet fibrinogen receptors are located on glycoprotein IIb/IIIa ($\alpha_{IIb}\beta_3$), the most abundant platelet integrin. It is well established that fibrinogen in solution does not bind with measurable affinity to resting platelets. However, it does bind specifically and saturably to activated platelets (Bennett and Vilaire, 1979; Marguerie et al., 1979). There is good evidence that platelet activation causes conformational changes within the $\alpha_{IIb}\beta_3$ complex itself, thereby inducing the formation of a ligand-binding pocket, or enabling access of ligand to a preformed binding pocket within the exoplasmic domain of the heterodimer (Sims et al., 1991). Several monoclonal antibodies recognize conformational changes in $\alpha_{IIb}\beta_3$ occurring during platelet activation (Shattil et al., 1987; Tomiyama et al., 1992a), or ligand binding (Frelinger et al., 1991; Kouns et al., 1991). In the present study, we have explored a possible application of disintegrins for the characterization of conformational changes in $\alpha_{IIb}\beta_3$.

In 1987, Huang et al. isolated trigramin, a potent inhibitor of fibrinogen binding to platelets and platelet aggregation that is found in the viper venom of *Trimeresurus gramineus* (Huang et al., 1987). Trigramin bound to $\alpha_{IIb}\beta_3$ receptors on resting platelets, but its binding affinity increased severalfold after platelet activation. Subsequently, a number of investigators isolated and characterized other trigramin-like molecules that have been named disintegrins (Chao et al., 1989; Dennis et al., 1990; Rucinski et al., 1990; Savage et al., 1990; Williams et al., 1990; Huang et al., 1991a,b; Scarborough et al., 1991, 1993). All disintegrins are known to contain at least one integrin-recognition site, an RGD/KGD sequence near the C-terminus (Gould et al., 1990; Scarborough et al., 1991). The activity of disintegrins depends on the appropriate pairing of eight to 14 cysteines by S–S bridges, which maintain the RGD-containing loop in an appropriate conformation (Calvete et al., 1991, 1992; Saudek et al., 1991; Adler et al., 1993). Disintegrins can bind to resting and to activated platelets, and the number of binding sites varies from 30000 to 50000 per platelet (Chao et al., 1989; Dennis et al., 1990; Savage et al., 1990; Huang et al., 1991a). Teng and Huang (1991) suggested that disintegrins may bind to platelets in two different patterns. Whereas flavoridin binds with the same affinity and to the same number of binding sites on resting and ADP-activated platelets (Huang et al., 1991b), trigramin (Huang et al., 1987), echistatin, applagin (Savage et al., 1990) and halysin (Huang et al., 1991a) bound to ADP-activated platelets with a 3- to 8-fold increased affinity compared with resting platelets.

We compared the interaction of four ^{125}I-labelled disintegrins (albolabrin, bitistatin, echistatin and eristostatin) and two fluorescein isothiocyanate (FITC)-labelled disintegrins (albolabrin and eristostatin) with resting platelets and platelets activated by ADP or thrombin. We also compared the binding affinities of these disintegrins with other properties of these compounds. These disintegrins exhibited significant heterogeneity in their interactions with $\alpha_{IIb}\beta_3$, especially when comparing resting and activated platelets.

MATERIALS AND METHODS

Lyophilized crude viper venoms were obtained from Latoxan (Rosan, France). Na^{125}I was from New England Nuclear (Boston, MA, U.S.A.) Iodobeads, bis(sulphosuccinimidyl) suberate (BS^3), and bicinchoninic acid (BCA) protein quantification kit were obtained from Pierce Chemical Co. Acrylamide/bis-acrylamide, TEMED, ammonium persulphate, SDS, 2-mercaptoethanol and protein molecular-mass-marker standards used in electrophoresis were from Bio-Rad. ADP, Sepharose CL-2B, X-Omat film and standard laboratory chemicals were obtained from Sigma Chemical Co. Trifluoroacetic acid and acetonitrile were purchased from Fisher Scientific. α-Thrombin

Abbreviations used: ACD, acid–citrate–dextrose; BS^3, bis(sulphosuccinimidyl)suberate; FITC, fluorescein isothiocyanate.

‡ To whom correspondence should be addressed.

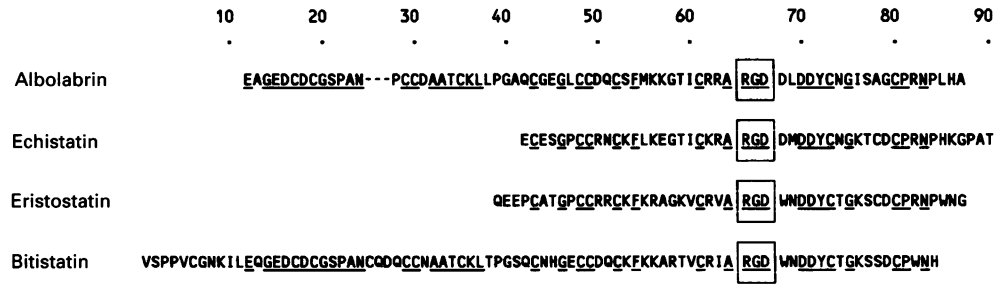

Figure 1 Amino acid sequences of disintegrins with invariant sequences underlined

Amino acids are represented by the single-letter code. Dashes represent a break in the sequence in order to align the N-terminal sequences optimally.

was obtained from the Division of Biological Standards (National Institutes of Health, Bethesda, MD, U.S.A.). Calbiochem supplied the fluorescein isothiocyanate–Celite (FITC). A Mono Q column was purchased from Pharmacia (Piscataway, NJ, U.S.A.), while the C-18 reverse-phase columns were from Vydac (Hesperia, CA, U.S.A.). The monoclonal antibodies OP-G2 and Ab62 were generous gifts from Dr. Thomas Kunicki (Blood Research Institute, Milwaukee, WI, U.S.A.) and Dr. Mark Ginsberg (Scripps Research Institute, La Jolla, CA, U.S.A.) respectively. Purified $\alpha_{IIb}\beta_3$ was a gift from Dr. Juan Calvete (Instituto de Quimica Fisica, 'Rocasolano', Madrid, Spain).

Purification and characterization of disintegrins

Bitistatin, echistatin and eristostatin [an isomorph of eristocophin isolated by Scarborough et al. (1991)] were purified to homogeneity from the crude venom of *Bitis arietans*, *Echis carinatus* and *Eristocophis macmahoni* respectively, using one- or two-step C-18 reverse-phase h.p.l.c. by the method of Williams et al. (1990). Albolabrin was purified from the venom of *Trimeresurus albolabris* by a combination of ion-exchange chromatography (Mono Q column) and C-18 reverse-phase h.p.l.c. (Lasz et al., 1993). The amino acid sequences of these four disintegrins are listed in Figure 1. The concentration of each disintegrin that inhibited platelet aggregation induced by 20 μM ADP was determined as described previously (Williams et al., 1990).

Preparation of human platelet suspensions

Aspirin-free blood was collected from healthy donors and from one patient with Glanzmann's thrombasthenia (Hematology Division, Christiana Medical Center, Christiana, DE, U.S.A.) in acid–citrate–dextrose (ACD) (final ratio 1:7, v/v) for flow cytometry studies; in ACD plus 15 units/ml heparin for radiolabelled binding studies; or in 3.8% (w/v) sodium citrate (1:9, v/v) for platelet-aggregation studies. Washed platelets were prepared according to the method of Mustard et al. (1972) and resuspended at $(3.0–5.0) \times 10^8$/ml in Tyrode's solution (134 mM NaCl, 3 mM KCl, 0.3 mM NaH_2PO_4, 2 mM $MgCl_2$, 5 mM Hepes, 0.89 M $NaHCO_3$, 5.5 mM glucose, 0.35% BSA, pH 7.35). Gel-filtered platelets for flow cytometry studies were prepared as described previously (Shattil et al., 1987) and were adjusted to 1.0×10^8/ml.

Binding of ^{125}I-disintegrins to washed platelets

This was measured by the method described by Niewiarowski et al. (1981) with minor modifications. Unless otherwise indicated, all bindings were performed at room temperature and for 5 min. In brief, purified disintegrins were radiolabelled with $Na^{125}I$ using Iodobeads. Specific radioactivities averaged 17.3–34.6 Bq/μg of peptide. To obtain binding isotherms, washed platelets were incubated with increasing concentrations of ^{125}I-labelled disintegrins (0.1–6.0 μg/ml), and then stimulated with ADP (60 μM) or α-thrombin (0.1 unit/ml). Non-specific binding under each condition was measured in the presence of 6 mM EDTA (which chelates calcium needed to maintain the $\alpha_{IIb}\beta_3$ complex in its optimal conformation) or bitistatin (50 molar excess), and was subtracted from total binding to calculate specific binding. Non-specific binding with EDTA amounted to 3–12% of total binding, while with bitistatin, it was 8–15% of total binding. After centrifuging through silicone oil (7500 *g*, 3 min), the platelet pellet and supernatant were counted separately in a γ-counter, with the data analysed according to the method of Scatchard (1949).

Statistical analysis

Binding data were analysed for statistical significance in two ways. Student's *t* test (two-tailed) for independent means and paired tests was used to compare one individual experiment with another, or two sets of activation conditions. The data were also pooled and the mean $\pm$95% confidence limits for binding sites and dissociation constants were calculated. For this, a computer program ('Analysis of Linear Regression') from the Manual of Pharmacologic Calculations with Computer Programs (Tallarida and Murray, 1986) was used.

FITC-labelling of disintegrins

The method utilized was that of Shattil et al. (1987). Briefly, albolabrin and eristostatin (500 μg of each) were dissolved in 1 ml of PBS/sodium carbonate, pH 8.8–9.0. FITC (500 μg) was added and incubated, with periodic mixing, for 60 min at room temperature. FITC-bound disintegrins were separated by C-18 reverse-phase h.p.l.c. Peptide concentration was determined by the Pierce protein microassay. The FITC:peptide ratio was 1.2:1. Platelet aggregation inhibitory activity (IC_{50}) of the FITC–disintegrins was identical with that of unlabelled peptide.

Analysis of disintegrin or anti-LIBS antibody binding by flow cytometry

Depending on the experiment, gel-filtered platelets (1×10^8/ml) were incubated at room temperature in the dark for 15 min with FITC-labelled albolabrin, eristostatin or the monoclonal anti-LIBS antibody (where LIBS is ligand induced binding site),

Table 1 Binding-site number and binding affinity of disintegrins to human resting and activated platelets

The number of binding sites and binding affinity for each disintegrin was estimated by Scatchard analysis of the ^{125}I binding data from three experiments performed in duplicate. Binding-site results are expressed as the mean and lower/upper 95% confidence limits (in parentheses). Binding-affinity data from these experiments are expressed in nmol/l, mean and lower/upper 95% confidence limits (in parentheses).

		Binding site number and binding affinity		
Ligand	Platelets...	Resting	ADP-activated	Thrombin-activated
Albolabrin		106397 (80727–132067)	92132 (76587–107676)	98404 (88477–108331)
		294 nM (219–447)	86 nM (66–123)*	48 nM (40–61)†
Eristostatin		58632 (40228–77037)	51328 (35943–66714)	76972 (51696–102248)
		18 nM (12–40)	12 nM (8–27)	15 nM (10–34)
Bitistatin		35291 (30677–39905)	32387 (29807–34967)	47358 (40800–53917)
		48 nM (40–62)	23 nM (19–27)‡	23 nM (18–32)
Echistatin		36407 (17901–54913)	44630 (32159–57101)	50793 (30089–71496)
		153 nM (92–473)	80 nM (57–153)§	63 nM (39–164)

* $P = 0.000093$, comparing resting versus ADP-activated; † $P = 0.0321$, comparing ADP- versus thrombin-activated; ‡ $P = 0.022$, resting versus ADP-activated; § $P = 0.0336$, resting versus ADP-activated; all other binding affinity P values > 0.05.

Ab62, in the presence of buffer containing ADP (60 μM), thrombin (0.1 unit/ml), EDTA (6 mM), unlabelled disintegrin or the monoclonal antibody OP-G2 (40 μg/ml). The reactions were stopped by adding a 10-fold vol. of PBS and samples were analysed by flow cytometry (Becton Dickinson FACStar, Braintree, MA, U.S.A.) (Shattil et al., 1987). Light scattering and fluorescence signals were analysed for 10000 platelets per sample. Results were expressed as mean platelet fluorescent intensity in arbitrary units.

Cross-linking of ^{125}I-disintegrins to $\alpha_{IIb}\beta_3$

All experiments were performed at room temperature without the addition of agonist. The cross-linker BS^3 was dissolved in dimethyl sulphoxide immediately before use. Washed platelets (3×10^8/ml) were incubated with saturating concentrations of radiolabelled disintegrins for 10 min, followed by incubation with 200 μM BS^3 for 20 min. The reaction was stopped by bringing the pH to 8.0. The samples were centrifuged and the pellets were boiled in 1 × Laemmli buffer under reducing conditions. Samples were then electrophoresed on SDS/7.5% polyacrylamide vertical slab gels (Laemmli, 1978). Gels were stained with Coomassie Blue, dried and exposed to Kodak X-Omat AR film for autoradiograms. To evaluate non-specific cross-linking, platelets were preincubated with 6 mM EDTA, 50 μg/ml bitistatin or the monoclonal antibody OP-G2 (40 μg/ml) before addition of the disintegrin or cross-linker. In addition, cross-linking was also performed using thrombasthenic platelets from a donor with a well-characterized $\alpha_{IIb}\beta_3$ content, which was known to be 3% of normal (Kornecki et al., 1981). In all cross-linking experiments, the radioactivity associated with the platelet pellet was also counted in a γ-counter.

RESULTS

Determination of the inhibitory effect of disintegrins on platelet aggregation (IC_{50})

The IC_{50} values (mean ± S.E.M.) for eristostatin ($n = 5$), bitistatin ($n = 7$), echistatin ($n = 11$) and albolabrin ($n = 30$) were 59 ± 22 nM, 139 ± 10 nM, 136 ± 29 nM and 185 ± 20 nM respectively. IC_{50} values for bitistatin and albolabrin were similar to those reported previously (Shebuski et al., 1989; Williams et al., 1990). The IC_{50} value for echistatin was higher than that estimated by Gan et al. (1988) and Musial et al. (1990), but lower than that reported by Dennis et al. (1990). The value for eristostatin has not been reported previously.

Binding of ^{125}I-labelled disintegrins to platelets

All four disintegrins bound to resting platelets and to platelets activated by 60 μM ADP or 0.1 unit/ml thrombin in a saturable manner. Table 1 summarizes the Scatchard analysis of all of the binding data and the tests for statistical significance. For each disintegrin, the observed differences in the number of binding sites between resting and activated platelets did not reach significance. Compared with the other disintegrins, under identical platelet activation conditions, the number of albolabrin-binding sites was about 2.5-fold higher in each instance.

Figure 2(a) shows the Scatchard analysis for albolabrin binding to resting, ADP- or thrombin-activated platelets. Figure 2(b) compares the Scatchard analyses for eristostatin, bitistatin and echistatin binding that were statistically different. The binding affinity of albolabrin increased 3-fold when platelets were stimulated by ADP and 6-fold following thrombin stimulation. These increases were significant. Bitistatin bound with a higher affinity to activated platelets than to resting platelets, and there was no difference in binding affinities between ADP- and thrombin-activated platelets. Although echistatin-binding data showed no difference if plotted by confidence limits, it did show a significant difference in binding affinity when comparing resting versus ADP-activated conditions by Student's t test. There was, however, no significant difference between echistatin binding affinity to ADP- and thrombin-activated platelets. Compared with albolabrin, echistatin and bitistatin, eristostatin had the highest binding affinity to resting platelets (18 nM), and this was not significantly influenced by platelet activation with ADP or thrombin (Table 1, Figure 2). Thus of the four disintegrins studied, albolabrin bound with the lowest affinity to resting platelets and its binding affinity increased progressively when platelets were activated by a 'weak' (ADP) and a strong (thrombin) agonist. Three disintegrins (albolabrin, echistatin and bitistatin) bound to thrombin-activated platelets with binding affinities that were 2- to 3-fold lower than eristostatin binding to resting or activated platelets. Preincubation of platelets with

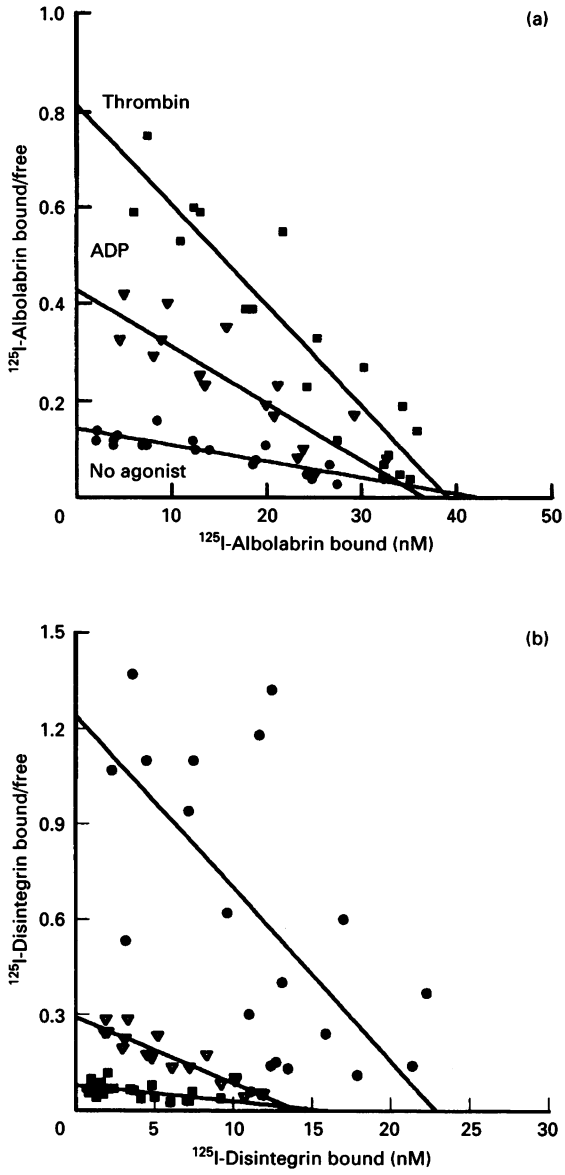

Figure 2 Scatchard analysis of ^{125}I-disintegrin binding to human resting and activated platelets

Washed platelets (3×10^8/ml) were incubated with increasing concentrations of ^{125}I-disintegrin, and binding was studied under three conditions: no agonist, ADP-activated (60 μM) or thrombin-activated (0.1 unit/ml). After 5 min, 400 μl of the platelet suspension were placed over silicone oil and centrifuged. Non-specific binding was measured after preincubation of platelets with 6 mM EDTA or a 50 molar excess of bitistatin before the addition of disintegrin or agonist, and was subtracted from all data. Binding isotherm data were analysed by the method of Scatchard. Each plot shows data from three experiments performed in duplicate. (**a**) Albolabrin, three activation conditions are shown; (**b**) eristostatin (●), bitistatin (▽), and echistatin (■), only results with resting platelets are shown.

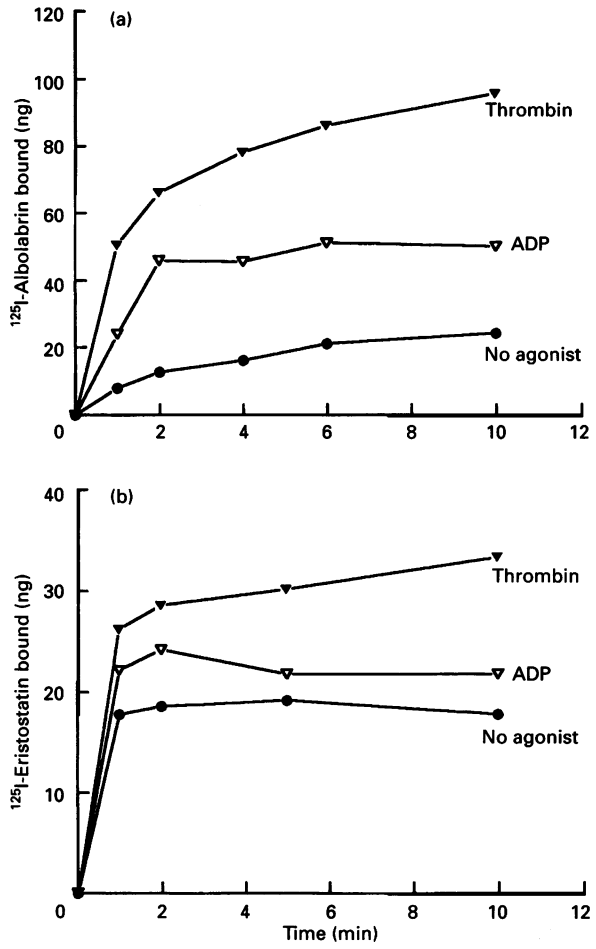

Figure 3 Effect of various incubation times on the binding of albolabrin and eristostatin to resting and activated platelets

Albolabrin (**a**) (1 μg/ml) or eristostatin (**b**) (0.5 μg/ml) were added to washed platelets (3×10^8/ml) in the absence or presence of ADP (60 μM) or thrombin (0.1 unit/ml). The mixture was incubated for varying intervals (1–10 min) at room temperature without stirring. After incubation 400 μl aliquots were placed over silicone oil and centrifuged. Supernatant and pellets were counted separately, and non-specific binding was subtracted from all data. Each graph is representative of three identical experiments performed in duplicate.

EDTA or a 50 molar excess of bitistatin resulted in non-specific binding of less than 15% of total binding.

In view of the observation that albolabrin and eristostatin showed very distinct binding patterns, their interactions with platelets were studied in greater detail. Figure 3 compares the time course of binding of low concentrations of albolabrin (1 μg/ml) and eristostatin (0.5 μg/ml) to resting and activated platelets. The rate of albolabrin binding was lower than that of eristostatin regardless of the degree of platelet activation. In fact, eristostatin binding reached steady state within 1 min, while albolabrin binding reached a plateau after 2 min.

Table 2 shows that, when 6 mM EDTA was added after 10 min of incubation, dissociation of albolabrin from platelets was temperature-dependent, with 58–75% of albolabrin dissociated at 25 °C and 37 °C, and 24% dissociated at 4 °C. Under the same conditions, less than 10% of eristostatin became dissociated from the platelets at any temperature. This experiment suggests that eristostatin, with a higher binding affinity to resting platelets than albolabrin, is more irreversibly bound. It also shows a difference in temperature dependence between binding of fibrinogen and disintegrin to platelets.

Binding of FITC–albolabrin and FITC–eristostatin to resting and activated platelets

Given the differences in affinities and number of binding sites among the disintegrins, the purpose of this series of experiments was to determine whether disintegrins bind homogeneously to all

Table 2 Irreversibility of ^{125}I-albolabrin or ^{125}I-eristostatin binding to ADP-activated platelets

^{125}I-albolabrin (1 μg/ml) or ^{125}I-eristostatin (0.5 μg/ml) and ADP (60 μM) were added concurrently to washed platelets and incubated for 10 min at 4 °C, 25 °C or 37 °C. After incubation, 400 μl aliquots were placed over silicone oil and centrifuged. In parallel experiments, EDTA (6 mM) was added to each sample at the conclusion of its timed incubation, before centrifugation. In the control experiments, non-specific binding was determined as usual, with preincubation of the platelets with 6 mM EDTA, and its subsequent subtraction to yield specific binding. Data are pooled from two experiments performed in duplicate, and expressed as the mean ± S.E.M. Percent decrease in binding was calculated using (control − EDTA)/control.

	Albolabrin bound (ng)			Eristostatin bound (ng)		
	4 °C	25 °C	37 °C	4 °C	25 °C	37 °C
Control	79 ± 2.8	126 ± 1.2	103 ± 5.2	33 ± 0.4	31 ± 0.1	29 ± 0.2
+ EDTA	60 ± 0.7	52 ± 3.1	25 ± 4.0	30 ± 1.2	29 ± 0.1	28 ± 0.8
Decrease in binding (%)	24	58	75	9	6	3

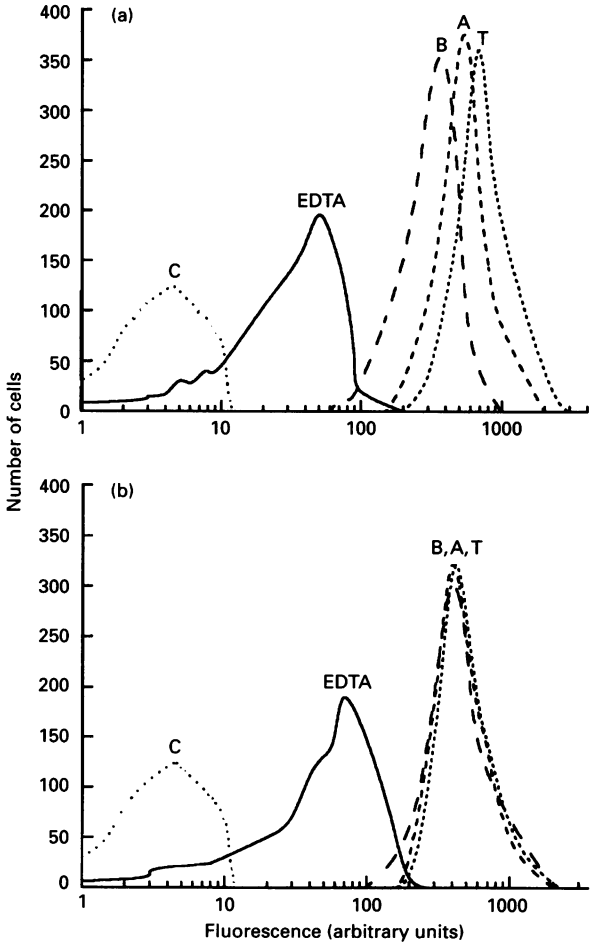

Figure 4 Analysis of FITC–disintegrin binding to platelets by flow cytometry

Gel-filtered platelets (1 × 10^8/ml) were incubated at room temperature in the dark for 15 min with (**a**) FITC–albolabrin (1.0 μg/ml) or (**b**) FITC–eristostatin (0.5 μg/ml) in the presence of buffer (B), ADP (60 μM) (A) or thrombin (0.1 unit/ml) (T). Reactions were stopped with PBS dilution, and the samples were analysed (10000 cells/sample) on an FACStar flow cytometer. Background autofluorescence of the platelets without FITC–disintegrin addition (C). Non-specific binding was measured in the presence of 6 mM EDTA, and was subtracted from total binding to give specific binding, shown in B, A and T. Each binding was repeated twice, in duplicate, with identical results.

populations of human platelets. Figure 4 shows flow-cytometric histograms illustrating the binding of FITC–albolabrin and FITC–eristostatin to resting and activated platelets. Pre-incubation of platelets with EDTA decreased the binding of either disintegrin at least 10-fold, thus giving consistent results with non-specific binding in the ^{125}I-disintegrin assay. ADP-activated platelets showed a 1.5-fold increase in FITC–albolabrin-dependent platelet fluorescence compared with resting platelets. The fluorescence intensity increased further when thrombin was the agonist. This binding pattern contrasts with FITC–eristostatin binding, in which the already high fluorescent signal generated with resting platelets did not increase further upon stimulation. In all cases, both disintegrins bound homogenously to the entire population of platelets. Thus the experiments with FITC-labelled disintegrins are consistent with the binding results obtained with ^{125}I-disintegrins, and demonstrate further that differences in the binding characteristics between albolabrin and eristostatin cannot be explained by heterogeneity of platelets with respect to disintegrin-binding sites.

Recently, Tomiyama et al. (1992a) described an RGD-dependent monoclonal antibody (OP-G2) directed against $\alpha_{IIb}\beta_3$ which binds to thrombin-stimulated platelets with a higher affinity than it does to resting platelets. An experiment was performed to assess whether this difference in binding affinity would affect the ability of this antibody to inhibit the binding of FITC–albolabrin or FITC–eristostatin to gel-filtered platelets. Compared with FITC–albolabrin alone, co-incubation of platelets with OP-G2 caused a 56% decrease in FITC–albolabrin binding to resting platelets and a 76% decrease in binding to ADP-activated platelets. On the other hand, OP-G2 had no effect on FITC–eristostatin binding to resting platelets, and decreased binding to ADP-stimulated platelets by only 16% (Table 3).

Induction of a 'ligand-induced binding site' epitope on platelet $\alpha_{IIb}\beta_3$ by disintegrins

We compared the ability of albolabrin and eristostatin to induce these neo-epitopes in gel-filtered platelets using FITC–Ab62, which is a mouse monoclonal anti-LIBS (anti-β_3) antibody (Frelinger et al., 1991). Figure 5 shows that both disintegrins (0.4–3.0 μg/ml) induced a dose-dependent expression of this LIBS epitope in resting platelets, with eristostatin being 30-fold more potent than albolabrin.

Cross-linking of ^{125}I-disintegrins to platelets

Figure 6(a) shows a Coomassie Blue-stained gel of purified $\alpha_{IIb}\beta_3$, and autoradiograms of platelets to which disintegrins have been cross-linked using BS^3. It can be seen that radioactivity was associated with platelet components co-migrating in the

Table 3 Effect of OP-G2 on the binding of FITC–disintegrins to gel-filtered platelets

Resting or ADP-activated gel-filtered platelets were incubated with saturating concentrations of FITC–disintegrin in the presence (+) or absence (−) of 50 μg/ml OP-G2. Non-specific binding (NSB) was measured with 6 mM EDTA. Data are expressed in arbitrary fluorescence units, and are representative of two identical experiments.

	Albolabrin			Eristostatin		
	NSB	Resting	ADP-activated	NSB	Resting	ADP-activated
−Antibody	37	297	780	68	805	813
+Antibody		131	191		804	685
Decrease in fluorescence (%)		56	76		0	16

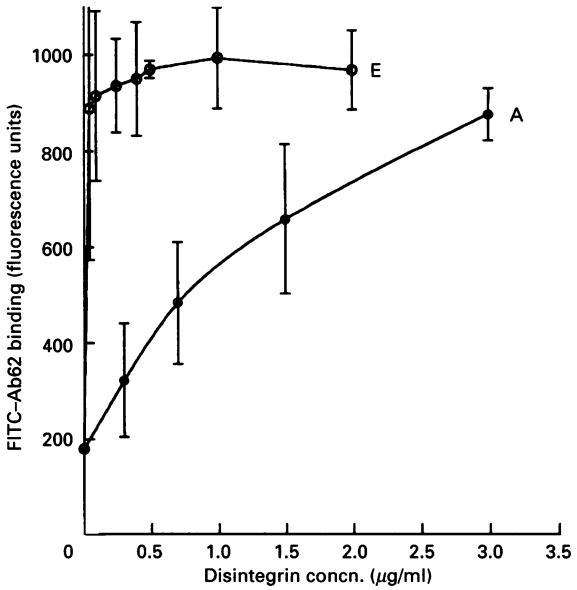

Figure 5 Analysis of ligand-induced binding sites on $\alpha_{IIb}\beta_3$ induced by disintegrins

Increasing concentrations of albolabrin (A) or eristostatin (E) were incubated for 15 min at room temperature with gel-filtered platelets (1×10^8/ml) in the presence of FITC–Ab62 (500 μg/ml), without agonist. Platelets were diluted with PBS and fluorescence measured by flow cytometry. Results are shown as the mean ± S.D. from four experiments.

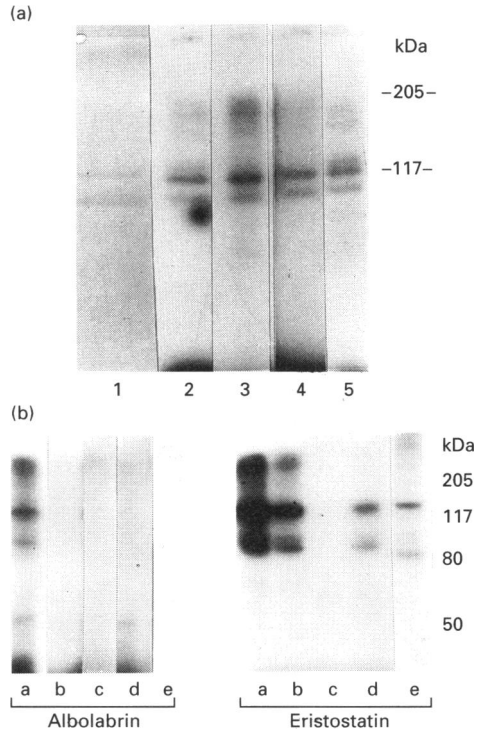

Figure 6 Cross-linking of ^{125}I-disintegrins to $\alpha_{IIb}\beta_3$

(**a**) Washed platelets (3×10^8/ml) were incubated with saturating concentrations of ^{125}I-disintegrins for 10 min, followed by incubation with the cross-linker BS^3 (200 μM) for 20 min. The reaction was stopped with 10 mM Tris (pH 8.0), and the samples were centrifuged. Pellets were resuspended in Laemmli buffer containing 2-mercaptoethanol (0.2%) and heated. Purified $\alpha_{IIb}\beta_3$, as a marker, was electrophoresed on an SDS/7.5% polyacrylamide gel and stained with Coomassie Blue (lane 1). Disintegrin autoradiograms: lane 2, albolabrin; lane 3, bitistatin; lane 4, echistatin; lane 5, eristostatin. (**b**) Washed normal platelets (a–d) were pre-incubated with buffer (a), 50 μg/ml bitistatin (b), 6 mM EDTA (c) or 40 μg/ml OP-G2 (d) for 10 min, then incubated with concentrations of ^{125}I-albolabrin or ^{125}I-eristostatin, and processed as described in (**a**) above. Thrombasthenic platelets (without agonist or inhibitors) were processed as usual, with results shown in lanes e. Each cross-linking was performed twice with identical results.

reduced system with the same mobility as isolated subunits of purified $\alpha_{IIb}\beta_3$. Similar results were obtained by analysing gels run in the non-reduced system (results not shown). In each instance, more ^{125}I-disintegrin was associated with α_{IIb} than with β_3. The ratio of radioactivity associated with α_{IIb} and β_3 (3:1) was similar for each disintegrin. Preincubation of platelets with EDTA, excess of bitistatin, or the anti-β_3 antibody OP-G2, before addition of radiolabelled albolabrin and cross-linker, resulted in a 100% decrease of radioactivity associated with $\alpha_{IIb}\beta_3$ (Figure 6b), indicating that the cross-linking reaction was specific. In the case of eristostatin, cross-linking was less inhibited by excess of bitistatin or OP-G2, which correlated with binding results, as eristostatin's binding affinity to resting platelets is greater than that of either bitistatin or the antibody. Pre-incubation of platelets with OP-G2 also resulted in the disappearance of the higher-molecular-mass bands to which eristostatin cross-linked. This suggests that such bands contain the β_3 subunit, perhaps dimerized by a cross-linking disintegrin molecule. In the case of albolabrin, there was more radioactivity associated with $\alpha_{IIb}\beta_3$ on activated than on resting platelets. No such differences were observed with eristostatin.

Since platelets contain low concentrations of RGD-dependent receptors for fibronectin and vitronectin (Thiagarajan and Kelly, 1988), we assessed the specificity of disintegrin interaction with $\alpha_{IIb}\beta_3$ by using platelets from a thrombasthenic individual known to be deficient in $\alpha_{IIb}\beta_3$ complex on their platelet surface

(Kornecki et al., 1981). There was no cross-linking of albolabrin when using these platelets, while for eristostatin there was a small amount of cross-linking seen. It is reasonable to propose that, with eristostatin's high binding affinity, its cross-linking to the small amount of $\alpha_{IIb}\beta_3$ complex (3% of normal) known to be present on this donor's platelets could still be visualized on the autoradiogram.

DISCUSSION

The present study shows that disintegrins can exhibit three different patterns of binding to platelets: (1) binding with the same binding affinity to resting and activated platelets (eristostatin); (2) binding with lower affinity to resting platelets than to activated platelets and no difference in affinity with weak or strong agonists (bitisatin and echistatin); and (3) having a binding affinity which correlates with the strength of the agonist: lowest with resting platelets, higher with a weak agonist, highest with a strong agonist (albolabrin). These characteristics of disintegrin binding to platelets were observed with either the radiolabelled or the FITC-labelled ligand. Binding studies with FITC-labelled ligand also demonstrated that the disintegrin-binding sites were homogeneously distributed on all platelets (Figure 4).

Several investigators have demonstrated cross-linking of short RGDX peptides to α_{IIb} and β_3 *in situ* using intact platelets (d'Souza et al., 1988). Sheu et al. (1992) showed cross-linking of the disintegrin, flavoridin, to the β_3 subunit on platelets. Cross-linking of ^{125}I-labelled disintegrins to platelets by means of the homobifunctional reagent BS^3 demonstrated a similar ratio of radioactivity between the components migrating on SDS/PAGE with apparent molecular masses of α_{IIb} and β_3, confirming its selective binding to $\alpha_{IIb}\beta_3$ (Figure 6). This pattern of cross-linking probably depends on the distribution of lysine residues on the surface of both components of the $\alpha_{IIb}\beta_3$ heterodimer in the vicinity of the putative disintegrin-binding site. The presence of a radioactive band with a molecular mass greater than 200 kDa suggests that the same disintegrin molecule may cross-link to both α_{IIb} and β_3. It is also possible that two β_3 subunits, cross-linked by a single disintegrin molecule, could have an apparent molecular mass of 180 kDa. Figure 6(b) shows that the anti-β_3 antibody OP-G2 (40 μg/ml) displaces disintegrin cross-linking, causing the disappearance of such higher-molecular-mass bands.

This study suggests a relationship between the binding affinities of disintegrins to resting platelets and the results of other tests of platelet–disintegrin interaction. For example, eristostatin had the highest binding affinity (Table 1) of the three disintegrins tested, and it exhibited: (1) the strongest inhibitory activity on platelet aggregation; (2) faster binding kinetics than albolabrin (Figure 3); (3) more irreversible binding to platelets as determined in displacement studies with EDTA (Table 2); and (4) 30-fold greater potency compared with albolabrin in inducing the conformational neo-epitope in β_3 recognized by the anti-LIBS antibody Ab62 (Figure 5). Albolabrin showed the lowest binding affinity with resting and ADP-activated platelets (Table 1), the highest IC_{50}, and was more easily dissociated by 6 mM EDTA (Table 2). Compared with albolabrin and eristostatin, bitistatin and echistatin showed both intermediate platelet inhibitory activity (IC_{50}) and intermediate binding affinity with ADP as platelet agonist (Table 1). These results suggest that many functional differences among the disintegrins may be ultimately related to their binding affinities.

The binding of albolabrin and eristostatin to platelets at various temperatures show some similarities and differences with fibrinogen binding to ADP-activated platelets. Fibrinogen binding to platelet $\alpha_{IIb}\beta_3$ becomes progressively irreversible with time, as shown by its persistent binding even in the presence of EDTA (Peerschke and Wainer, 1985). In the present study, it was found that 75% of bound albolabrin was displaced at 37 °C, while at 4 °C, most of the albolabrin remained bound (Table 2). While eristostatin did resemble fibrinogen's irreversibility at the higher temperatures, at 4 °C little was displaced. The observations do suggest that irreversibility of disintegrin binding may be related to their binding affinities for resting platelets.

The monoclonal antibody OP-G2 binds with an increasing affinity to activated platelets in an RGD-dependent manner (Tomiyama et al., 1992b). The K_d of OP-G2 for resting platelets was estimated to be 25 nM (Tomiyama et al., 1992a), which was sufficient to inhibit FITC–albolabrin binding to resting platelets ($K_{d_{rest}} = 294$ nM), but not to prevent FITC–eristostatin ($K_{d_{rest}} = 18$ nM) from binding (Table 3). The higher binding affinity of the antibody for thrombin-stimulated platelets (5 nM) could explain why it was able to inhibit both disintegrins ($K_{d_{albo}} = 48$ nM; $K_{d_{eristo}} = 15$ nM) from binding to ADP-stimulated platelets, with albolabrin inhibited more than eristostatin. This may reflect the changes in conformational 'fit' which occur during platelet activation, and to which disintegrins and antibodies like OP-G2 are sensitive. The binding pattern of this antibody to platelets and its RGD-dependence (Tomiyama et al., 1992b) suggest the same site or spatially close recognition site(s) to that of the disintegrins.

We propose that the disintegrin-recognition site may be converted from a low- into a high-affinity state upon conformational changes in the $\alpha_{IIb}\beta_3$ receptor. Our results suggest that thrombin is inducing more extensive conformational changes than ADP as albolabrin bound to thrombin-activated platelets with higher affinity than to ADP-activated platelets. The increase of albolabrin binding affinity following platelet activation by ADP and thrombin may reflect increased availability of a site located on the adhesive domain of β_3 (Lasz et al., 1993).

This study also contributes to an understanding of the structure–function relationship of disintegrins. It has been suggested that the substitution of a hydrophilic amino acid such as aspartic acid (D) in the position adjacent to the C-terminal end of RGD with a hydrophobic residue such as phenylalanine (F) or tryptophan (W) enhances severalfold the ability of disintegrins to inhibit platelet aggregation and binding of fibrinogen to purified $\alpha_{IIb}\beta_3$ integrin (Huang et al., 1991b; Teng and Huang, 1991; Scarborough et al., 1993). This is consistent with our observations that albolabrin and echistatin (RGDD sequence) bind with lower affinity to resting and to activated platelets than bitistatin and eristostatin (RGDW sequence). However, eristostatin binds with higher affinity to resting platelets than bitistatin, and echistatin binds to resting platelets with higher affinity than albolabrin. It is conceivable that differences between secondary structure of the disintegrins (Calvete et al., 1992) or sequences other than RGDX (Wright et al., 1993) are involved in the determination of disintegrin specificity and potency.

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