Immunological characterization of eristostatin and echistatin binding sites on $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ integrins

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Two disintegrins with a high degree of amino acid sequence similarity, echistatin and eristostatin, showed a low level of interaction with Chinese hamster ovary (CHO) cells, but they bound to CHO cells transfected with $\alpha_{IIb}\beta_3$ genes (A5 cells) and to CHO cells transfected with $\alpha_v\beta_3$ genes (VNRC3 cells) in a reversible and saturable manner. Scatchard analysis revealed that eristostatin bound to 816000 sites per A5 cell (K_d 28 nM) and to 200000 sites (K_d 14 nM) per VNRC3 cell respectively. However, VNRC3 cells did not bind to immobilized eristostatin. Echistatin bound to 495000 sites (K_d 53 nM) per A5 cell and to 443000 sites (K_d 20 nM) per VNRC3 cell. As determined by flow cytometry, radiobinding assay and adhesion studies, binding of both disintegrins to A5 cells and resting platelets and binding of echistatin to VNRC3 cells resulted in the expression of ligand-induced binding sites (LIBS) on the β_3 subunit. Eristostatin inhibited, more strongly than echistatin, the binding of three monoclonal antibodies: OPG2 (RGD motif dependent), A2A9 ($\alpha_{IIb}\beta_3$ complex dependent) and 7E3 ($\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ complex dependent) to A5 cells, to resting and to activated platelets and to purified $\alpha_{IIb}\beta_3$. Experiments in which echistatin and eristostatin were used alone or in combination to inhibit the binding of 7E3 and OPG2 antibodies to resting platelets suggested that these two disintegrins bind to different but overlapping sites on $\alpha_{IIb}\beta_3$ integrin. Monoclonal antibody LM 609 and echistatin seemed to bind to different sites on $\alpha_v\beta_3$ integrin. However, echistatin inhibited binding of 7E3 antibody to VNRC3 cells and to purified $\alpha_v\beta_3$, suggesting that $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ might share the same epitope to which both echistatin and 7E3 bind. Eristostatin had no effect in these systems, providing further evidence that it binds to a different epitope on $\alpha_v\beta_3$.

INTRODUCTION

Disintegrins are a family of naturally occurring, cysteine-rich polypeptides that potently inhibit platelet aggregation and cell adhesion by interacting with such integrins as $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$ and $\alpha_5\beta_1$ (reviewed in [1–3]). So far about 30 disintegrins have been isolated and characterized from the venom of many species of snakes. These disintegrins contain 49–84 amino acids and 8–14 cysteine residues linked by intramolecular disulphide bonds; disintegrin activity disappears after reduction [4]. The biological activity of disintegrins depends on the structure of an RGD-containing loop maintained in an appropriate conformation by disulphide bridges. There is evidence that amino acids adjacent to RGD and amino acids in the C-terminal disintegrin region contribute to the modulation of disintegrin activity [5,6]. There is also evidence that various disintegrins show a high degree of selectivity with respect to their interactions with various integrins; for example bitistatin, barbourin and eristostatin are quite selective inhibitors of $\alpha_{IIb}\beta_3$, whereas echistatin is a more potent inhibitor of ligand binding to purified $\alpha_v\beta_3$ and $\alpha_5\beta_1$ than to purified $\alpha_{IIb}\beta_3$ [7]. Disintegrins can also differentiate between resting and activated receptors; for example eristostatin binds with the same affinity to resting and to activated platelets, whereas the binding affinity of echistatin for platelets increases severalfold after activation of $\alpha_{IIb}\beta_3$ [8].

Despite functional differences, the polypeptide chains of echistatin and eristostatin have the same number of amino acids, 49, and show 60 % amino acid sequence identity. Both disintegrins have cysteine residues at the same positions and they seem to have an identical pattern of disulphide bridges [9,10]. Because the structure of both disintegrins is well characterized they can be used as molecular probes to characterize ligand binding sites on integrins.

Integrins $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ possess a β subunit with an identical polypeptide chain and constitute the cytoadhesin family [11]. The α subunits of cytoadhesins are synthesized by a common mechanism and possess 74 % amino acid sequence similarity in their polypeptide chains [12]. The fibrinogen receptor ($\alpha_{IIb}\beta_3$ integrin) was found mainly in platelets [13], a few kinds of tumour cell [14] and cells of megakaryocyte lineage [15], but vitronectin receptor ($\alpha_v\beta_3$ integrin) is more broadly distributed in tissues. It occurs in endothelial cells [16], monocytes [17], osteoclasts [18] and melanoma cells [19]. A small amount of $\alpha_v\beta_3$ (approx. one-thousandth of the amount of $\alpha_{IIb}\beta_3$) was found on the surface of platelets [20,21]. $\alpha_{IIb}\beta_3$ is the most extensively investigated integrin, and a structural model of this integrin has been proposed [22]. It is known that $\alpha_{IIb}\beta_3$ changes its conformation and expresses new antigenic sites after ligand binding [23]. These epitopes can be detected by anti-LIBS monoclonal antibodies against ligand-induced binding sites (LIBS). Anti-LIBS antibodies recognize epitopes on the α [24] or β subunits [25,26].

In the present study, we characterized echistatin and eristostatin binding sites on $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ integrins with the aid of monoclonal antibodies recognizing ligand binding sites, LIBS on the β_3 subunit of the integrins, and $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ complex-

Abbreviations used: CHO, Chinese hamster ovary; CMFDA, 5-chloromethylfluorescein diacetate; FITC, fluorescein isothiocyanate; HBSS, Hanks balanced salt solution; LIBS, ligand-induced binding sites; PRP, platelet-rich plasma.

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dependent epitopes. We investigated the induction of the expression of LIBS epitope by disintegrins by using anti-LIBS2 monoclonal antibody Mab62 [27], which recognizes an epitope near the C-terminal end of the β_3 subunit [28].

MATERIALS AND METHODS

Proteins, peptides and antibodies

Eristostatin and echistatin were purified to homogeneity from the crude venoms of *Eristocophis macmahoni* and *Echis carinatus* respectively, by two-step C_{18} reverse-phase HPLC [29].

The following monoclonal antibodies were used as purified IgG: OPG2 and AP7, which recognize the RGD binding site on $\alpha_{IIb}\beta_3$ integrin, were made as described [30,31]; A2A9, provided by Dr. J. Bennett (University of Pennsylvania, Philadelphia, PA, U.S.A.), is dependent on $\alpha_{IIb}\beta_3$ complex [32]; 7E3, obtained from Dr. B. Coller (Mt. Sinai Hospital, New York, NY, U.S.A.), recognizes $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ complexes [33]; Mab62, provided by Dr. M. Ginsberg (Scripps Research Institute), is specific for LIBS2 [27]; and LM 609, obtained from Dr. D. Cheresh (Scripps Research Institute), recognizes the $\alpha_v\beta_3$ complex [34].

Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG was purchased from Jackson ImmunoResearch (West Grove, PA, U.S.A.). GRGDSPK peptide was synthesized by Dr. B. Jameson (Thomas Jefferson University, Philadelphia, PA, U.S.A.). CNBr-Sepharose 4B and 5-chloromethylfluorescein diacetate (CMFDA) were purchased from Pharmacia (Piscataway, NJ, U.S.A.) and Molecular Probes (Eugene, OR, U.S.A.) respectively. $Na^{125}I$ and Bolton–Hunter reagent ^{125}I-labelling kit were acquired from ICN Radiochemicals (Irvine, CA, U.S.A.). n-Octylglucoside and Hanks balanced salt solution (HBSS) were obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.) and Gibco BRL (Gaithersburg, MD, U.S.A.) respectively. All other reagents were purchased from Sigma (St. Louis, MO, U.S.A.).

Cell culture

A5 and VNRC3 cells, Chinese hamster ovary (CHO) cells expressing human $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ respectively [35,36], were kindly provided by Dr. M. Ginsberg. Non-transfected CHO-K1 cells were purchased from ATCC (Rockville, MD, U.S.A.). A5 and VNRC3 cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum, non-essential amino acids, glutamine, penicillin and streptomycin. CHO-K1 cells were maintained in Ham's F12 medium containing 10% (v/v) fetal calf serum, glutamine, penicillin and streptomycin. Cells were detached from plates with cation-free HBSS and 5 mM EDTA, and washed three times with HBSS containing Ca^{2+} and Mg^{2+}.

Purification of $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ integrins

Vitronectin receptor $\alpha_v\beta_3$ was purified to homogeneity by the method of Pytela et al. [37] with our own modifications. Briefly, VNRC3 cells, which express human $\alpha_v\beta_3$, were used as the source of integrin. Approx. 2×10^8 cells were lysed in the presence of 8 ml of buffer A (50 mM β-D-octylglucoside, 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 1 mM PMSF). After removal of insoluble material by centrifugation at 3000 ***g*** for 15 min at 5 °C, the extract was concentrated to 1 ml with Centriprep 10 (Amicon, Beverly, MA, U.S.A.) and then applied to a GRGDSPK–Sepharose 4B column at ambient temperature. The column was incubated for 60 min to increase the binding of integrin to the resin. The column was washed with buffer A and then with a modified buffer A containing 300 mM NaCl and no PMSF. The receptor was eluted by adding 15 mM EDTA to the modified buffer A, and 1 ml fractions were collected in vials containing 30 μl of 1 M $MgCl_2$. Fractions containing proteins were combined, concentrated to about 1 ml and dialysed against buffer A. Purified $\alpha_v\beta_3$ was stored at -20 °C in small aliquots.

Fibrinogen receptor $\alpha_{IIb}\beta_3$ was purified from outdated platelets (4 days in storage) as described [38].

Preparation of gel-filtered platelets

Platelets were isolated from fresh human blood treated with acid citrate dextrose anticoagulant. In some experiments, 1 μg/ml prostaglandin E1 was added with anticoagulant to prevent platelet activation during preparation. Gel-filtered platelets were obtained as described [6].

Binding of ^{125}I-labelled disintegrins and ^{125}I-labelled Mab62 to CHO cells and platelets

Disintegrins were labelled with ^{125}I by using Bolton–Hunter reagent in accordance with the original method [39] and the manufacturer's reccomendations. This method of radiolabelling did not alter the disintegrins' biological activity as tested in the platelet aggregation inhibition assay. In contrast, we observed that ^{125}I radiolabelling with the Pierce iodobeads resulted in a decrease of biological activity of disintegrins. Mab62 monoclonal antibody (IgG) was radiolabelled with chloramine T by the procedure described previously [40].

Binding of radiolabelled disintegrins to CHO cells was performed with the procedure for platelets [41] with some modifications. Briefly, the cells (7×10^5) were incubated with ^{125}I-labelled disintegrin in HBSS buffer containing 1% (w/v) BSA at room temperature for 15 min. The cells were separated by centrifugation through silicone oil (7500 ***g*** for 3 min), and the pellet and supernatant were counted separately in a γ-counter. Results were analysed by the method of Scatchard [42]. Non-specific binding under each condition was measured in the presence of a 50-fold molar excess of unlabelled disintegrin.

Binding of ^{125}I-labelled Mab62 IgG was performed under the same conditions for platelets and for CHO cells. The cells were preincubated with disintegrin (or with buffer as control) for 15 min at room temperature. ^{125}I-labelled Mab62 was then added, and the incubation continued for 45 min. Data were analysed as described above. Non-specific binding was measured in the presence of a 50-fold excess of unlabelled Mab62.

Flow cytometry analysis

Analyses with CHO cells were performed with 5×10^5 cells per sample. Cells were incubated with disintegrins for 15 min at room temperature. Monoclonal antibodies (1 μg per sample) were added for 45 min at 4 °C, and subsequently FITC-conjugated goat anti-mouse IgG (1 μg per sample) was added for 45 min at 4 °C. After each incubation step, the cells were washed with HBSS containing 1% (w/v) BSA. For platelets, 6×10^6 cells were used per sample, and incubations with monoclonal antibodies and FITC-conjugated goat anti-mouse IgG were performed at room temperature for 30 min in Tyrode's buffer, pH 7.4. Finally, both kinds of cell were fixed with 1% (w/v) paraformaldehyde. Analysis was done with a Coulter Epics Elite flow cytometer (Miami, FL, U.S.A.). Non-specific fluorescence was evaluated by measuring the binding of FITC-conjugated goat anti-mouse IgG in the absence of a primary monoclonal antibody.

The experiments with FITC–disintegrins were performed in a similar manner. Non-specific fluorescence was evaluated by measuring the binding of FITC–BSA (1 μg per sample). Disintegrins were labelled with FITC as described previously [8].

Statistical analysis

Results were analysed for statistical significance by using Student's *t*-test for independent means to compare one individual experiment with another. The results were also pooled and the means $\pm$ 95 % confidence limits were calculated as described previously [8].

Adhesion studies

Each well of the 96-well microplates (Costar) was coated with 10 μg of disintegrins or monoclonal antibodies in 50 mM carbonate/bicarbonate buffer, pH 9.2, and incubated at 4 °C overnight. The plate was blocked with 1 % (w/v) BSA in HBSS and stored at 4 °C until needed.

CHO cells, at a concentration of 5×10^6 cells/ml, were suspended in HBSS containing 1 % (w/v) BSA and 12.5 μM CMFDA and incubated at 37 °C for 20 min. The labelled cells were washed three times with HBSS containing 1 % BSA to remove excess CMFDA.

CMFDA-labelled cells (5×10^5) were added to each well of the plates and incubated at 37 °C for 15 min. The wells were washed four times with 1 % (w/v) BSA in HBSS. Cells that had adhered to the wells were lysed by the addition of 0.5 % Triton X-100. The amount of fluorescence associated with the wells was quantified with a Cytofluor 2350 fluorescence plate reader (Millipore, Bedford, MA, U.S.A.). In parallel, serial dilutions of CMFDA-labelled CHO cells were prepared in the same plate to make a standard curve from which the number of adhered cells was calculated.

ELISA

Each well of the microplates (Costar) was coated with 300 ng of purified integrin in 0.05 M carbonate/bicarbonate buffer, pH 9.2, and incubated at 4 °C overnight. To block non-reacted surfaces, the plates were incubated for 60 min at 37 °C with PBS/0.5 % Tween 20 containing 5 % (v/v) non-fat milk. After washing three times with PBS/Tween, various concentrations of disintegrin in a buffer containing 20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 % BSA were added and incubated at ambient temperature for 30 min. After washing with PBS/Tween, monoclonal antibodies were added for 60 min at 37 °C. After washing, the binding of monoclonal antibodies to integrins was detected with alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO, U.S.A.) as described previously [38].

RESULTS

Binding of eristostatin and echistatin to A5 and VNRC3 cells

Previous experiments from our laboratory indicated that eristostatin binds with a high affinity and quite selectively to $\alpha_{IIb}\beta_3$ integrin [7,8] whereas echistatin is a stronger inhibitor of ligand binding to purified $\alpha_v\beta_3$ than to purified $\alpha_{IIb}\beta_3$ [7]. To further compare the interaction of both disintegrins with $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$, we studied the binding of ^{125}I-labelled disintegrins to CHO cells transfected with either $\alpha_{IIb}\beta_3$ or $\alpha_v\beta_3$. Both echistatin and eristostatin showed a low level of interaction with CHO cells, but they bound to CHO cells transfected with $\alpha_{IIb}\beta_3$ genes (A5 cells) and to CHO cells transfected with $\alpha_v\beta_3$ genes (VNRC3 cells) in a reversible and saturable manner. Scatchard analysis (Table 1 and Figure 1) revealed that both disintegrins bound with a high affinity to 230000–820000 sites on both types of transfected cell, whereas the number of binding sites on non-transfected cells was approx. 40000. The number of eristostatin binding sites on A5 cells was over 60 % higher than the number of echistatin binding sites. On VNRC3 cells the number of echistatin binding sites was almost double the number of eristostatin binding sites. FITC–eristostatin also bound much more extensively to A5 cells than to VNRC3 cells, whereas FITC–echistatin bound efficiently to both types of cells (results not shown).

Table 1 Number of binding sites and affinity of disintegrins for CHO cells

The numbers of binding sites and binding affinity for eristostatin and echistatin were estimated by Scatchard analysis of the ^{125}I binding data from three experiments. The results correspond to means $\pm$ S.D.

Disintegrin	A5 cells	VNRC3 cells	CHO-K1 cells
Eristostatin			
Binding sites	816700 $\pm$ 78300	237530 $\pm$ 32640	37740 $\pm$ 2560
Binding affinity (nM)	28.26 $\pm$ 3.99	14.13 $\pm$ 1.13	9.6 $\pm$ 2.3
Echistatin			
Binding sites	495500 $\pm$ 41800	442920 $\pm$ 21400	41650 $\pm$ 3650
Binding affinity (nM)	53.51 $\pm$ 16.19	20.29 $\pm$ 3.54	13.58 $\pm$ 2.22

A5 cells adhered very well (approx. 47 % of total cell input) to immobilized eristostatin but adhesion of VNRC3 cells to immobilized eristostatin was at the level of control binding to BSA (approx. 3 % of total cell input). On the other hand, approx. 38 % of A5 cells and approx. 34 % of VNRC3 cells adhered to immobilized echistatin.

Exposure of LIBS2 epitope by disintegrins

The different reactivities of both disintegrins with $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ were further investigated by studying their effect on the expression of the LIBS epitope on the β_3 integrin subunit that is recognized by the monoclonal antibody Mab62. Figure 2A shows that both eristostatin and echistatin induced LIBS epitope on A5 cells in a dose-dependent manner as determined by flow cytometry. However, eristostatin was a more potent LIBS inducer than echistatin because a lower concentration was required for maximum expression of LIBS epitope. Echistatin induced LIBS epitope expression on VNRC3 cells, whereas the effect of eristostatin on LIBS epitope expression on VNRC3 cells was negligible. A similar observation has been made by studying the effect of these disintegrins on the adhesion of A5 and VNRC3 cells to immobilized Mab62 (Figure 2D). Binding of ^{125}I-labelled Mab62 IgG to A5 cells (Figure 2B) and to platelets (Figure 2C) was stimulated by eristostatin and echistatin in a dose-dependent manner. Interestingly, eristostatin also enhanced Mab62 binding to VNRC3 cells (Figure 2B), although to a much smaller extent than echistatin. Table 2 shows the number of binding sites and the binding affinity of Mab62 to transfected cells and to platelets in the absence and presence of 100 nM disintegrins. It can be seen that disintegrins, at concentrations approaching saturation, caused a significant increase in the binding of Mab62 to transfected cells and platelets. The level of induction of LIBS2 binding sites by eristostatin on VNRC3 cells was low but statistically significant (Table 2).

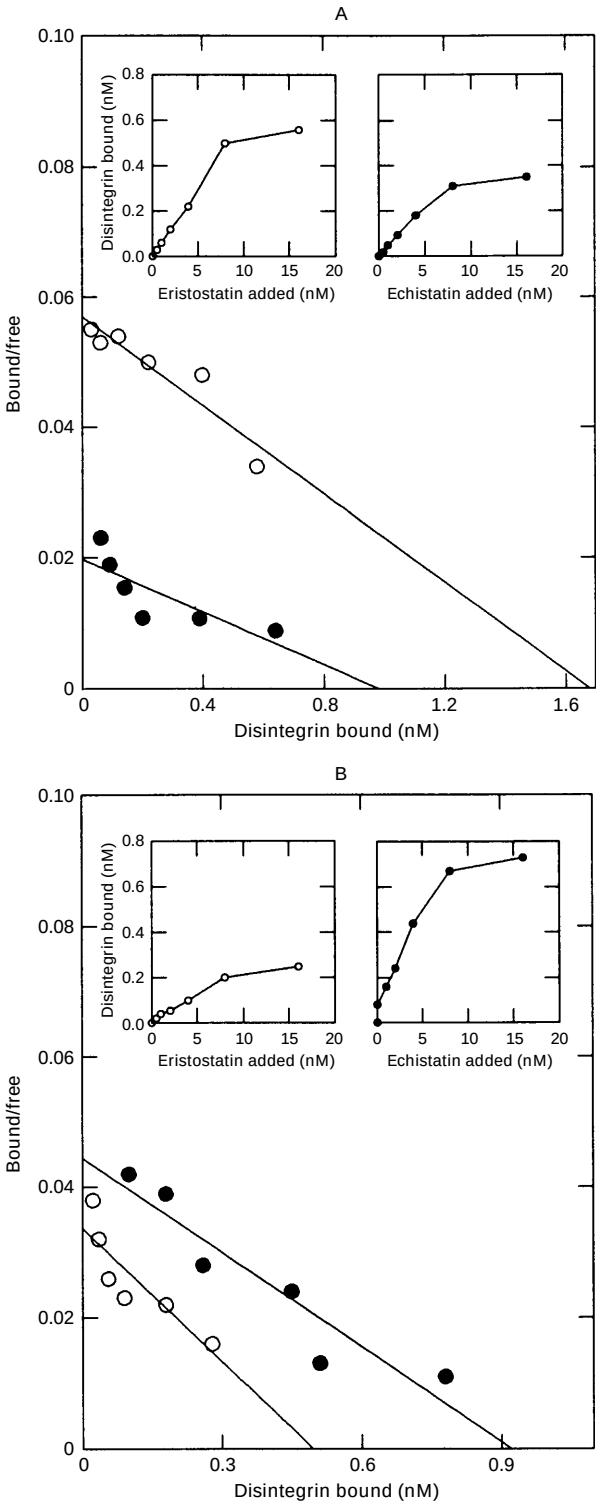

Figure 1 Scatchard analysis of ^{125}I-labelled disintegrins binding to A5 cells (A) and to VNRC3 cells (B)

Cells (5×10^5) were incubated with increasing concentrations of ^{125}I-labelled eristostatin (○) or ^{125}I-labelled echistatin (●) for 15 min at 37 °C, and then 400 μl of cell suspension was placed over silicone oil and centrifuged. The radioactivities of supernatant and pellet were measured with a γ-counter. Non-specific binding was measured after preincubation of cells with a 50-fold molar excess of the same unlabelled disintegrin. The results are means from three experiments.

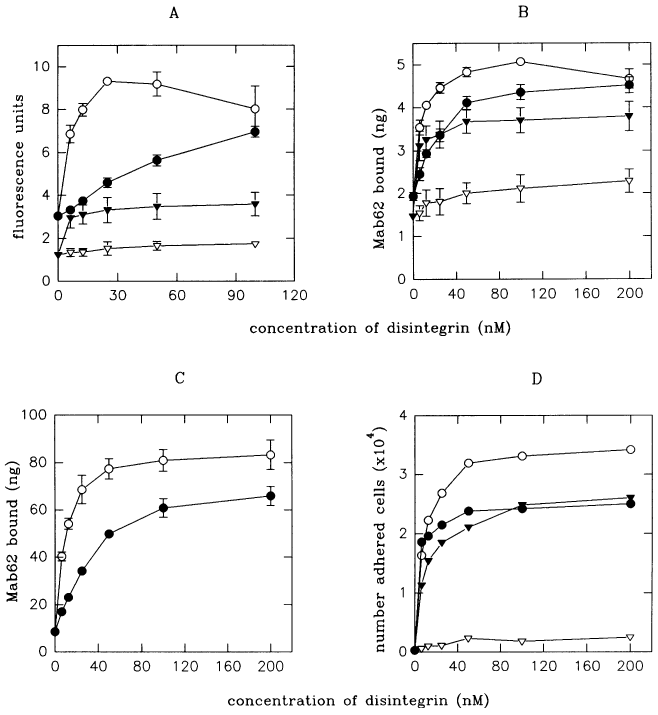

Figure 2 Exposure of LIBS2 epitope by eristostatin and echistatin

(**A**) A5 cells (○, ●) or VNRC3 cells (▽, ▼) (5×10^5 of each) were incubated with either eristostatin (open symbols) or echistatin (filled symbols) for 15 min at room temperature. After being washed, Mab62 (1 μg per sample) was added for 45 min at 4 °C. Cells were then incubated with FITC-conjugated goat anti-mouse IgG, fixed and analysed by flow cytometry as described in the Materials and methods section. Values represent the means ± S.D. for triplicate samples. (**B**) A5 cells (○, ●) or VNRC3 cells (▽, ▼) (5×10^5 of each) were preincubated with eristostatin (open symbols) or echistatin (filled symbols) for 15 min at room temperature. After being washed, ^{125}I-labelled Mab62 (1 μg per sample) was added and incubation was continued for 45 min. The pellet was separated from the supernatant by centrifugation through silicone oil and analysed as described in the Materials and methods section. Values represent means ± S.D. for triplicate samples. (**C**) The experiment was performed under the same conditions as for (**B**) with 6.7×10^7 gel-filtered platelets instead of transfected cells. (**D**) CMFDA-labelled A5 cells (○, ●) or VNRC3 cells (▽, ▼) (5×10^5 per sample) were preincubated with eristostatin (open symbols) or echistatin (filled symbols) for 15 min at room temperature and then added to immobilized Mab62 and incubated for 30 min at 37 °C. The numbers of adhered cells were calculated as described in the Materials and methods section.

Table 2 Number of binding sites and binding affinity of Mab62 monoclonal antibody for CHO cells and human resting platelets in the absence or presence of disintegrins

The number of binding sites and binding affinity for Mab62 were estimated in the absence or presence of disintegrin at a concentration of 100 nM by Scatchard analysis of the ^{125}I binding data from three experiments. The results correspond to means ± S.D. $P = 0.000006$ and 0.00025 comparing A5 cells without disintegrins and A5 cells with eristostatin and echistatin respectively; $P = 0.0013$ and 0.00013 comparing VNRC3 cells without disintegrins and VNRC3 cells with eristostatin and echistatin respectively; $P = 0.0000004$ and 0.0005 comparing platelets without disintegrins and platelets with eristostatin and echistatin respectively.

Disintegrin	A5 cells	VNRC3 cells	Platelets
None			
Binding sites	18130 ± 2220	14020 ± 1300	890 ± 160
Binding affinity (nM)	3.16 ± 0.96	5.32 ± 1.14	15.74 ± 3.54
Eristostatin			
Binding sites	47850 ± 1510	20140 ± 1330	10490 ± 200
Binding affinity (nM)	4.45 ± 1.20	4.64 ± 0.64	14.77 ± 2.23
Echistatin			
Binding sites	36770 ± 2950	34370 ± 3970	6860 ± 500
Binding affinity (nM)	3.92 ± 2.53	3.2 ± 0.41	12.76 ± 0.14

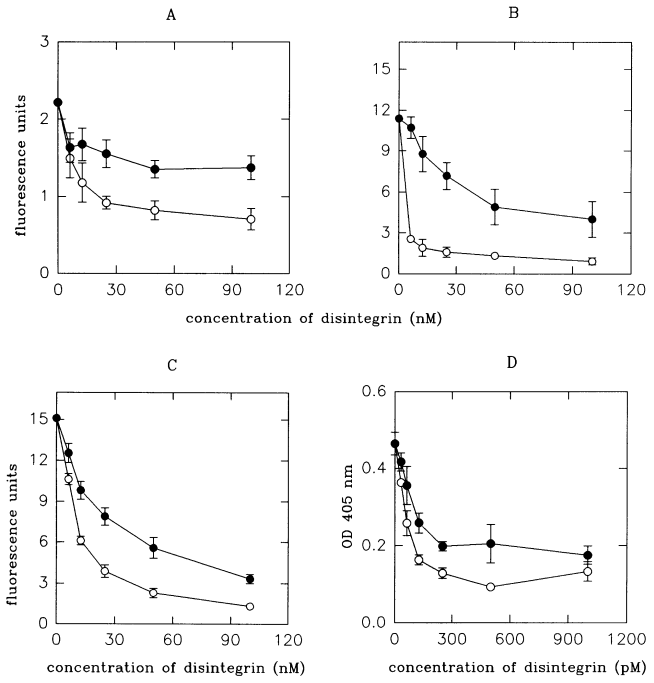

Figure 3 Effect of eristostatin (○) and echistatin (●) on binding of monoclonal antibody OPG2 to $\alpha_{IIb}\beta_3$ integrin

Flow cytometry was performed with A5 cells (**A**), resting platelets (**B**) or ADP-activated platelets (**C**). The cells were incubated with disintegrins for 15 min at room temperature and then with OPG2 (1 μg per sample) for 45 min at 4 °C for A5 cells, or for 30 min at room temperature for platelets. The level of bound OPG2 was detected with FITC-conjugated goat anti-mouse IgG as described in the Materials and methods section. Values represent means ± S.D. for triplicate samples. (**D**) ELISA assay was performed to measure the binding of OPG2 to immobilized $\alpha_{IIb}\beta_3$ (300 ng per well) in the presence of disintegrins. The amount of bound OPG2 was detected with alkaline phosphatase-conjugated goat anti-mouse IgG as described in the Materials and methods section. Error bars represent the S.D. for five independent experiments.

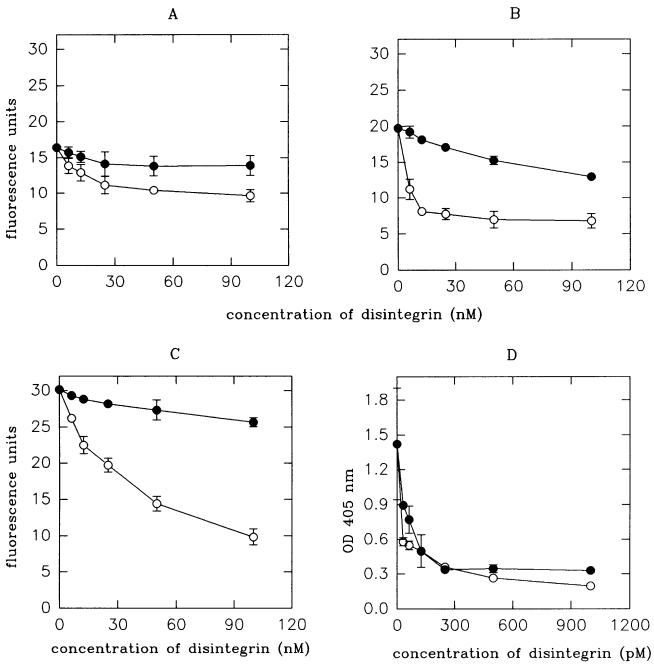

Figure 4 Effect of eristostatin (○) and echistatin (●) on binding of monoclonal antibody A2A9 to $\alpha_{IIb}\beta_3$ integrin

Flow cytometry using A5 cells (**A**), resting platelets (**B**) and ADP-activated platelets (**C**) and an ELISA assay (**D**) with immobilized $\alpha_{IIb}\beta_3$ were performed as described in the caption to Figure 3 except that A2A9 was substituted for OPG2.

Inhibitory effect of disintegrins on binding of OPG2 to $\alpha_{IIb}\beta_3$ integrin

Monoclonal antibody OPG2 binds to A5 cells, to platelets and to immobilized $\alpha_{IIb}\beta_3$ (Figure 3) but it does not bind to VNRC3 cells and to immobilized $\alpha_v\beta_3$ (results not shown). This antibody recognizes the RGD binding site on the β_3 subunit of $\alpha_{IIb}\beta_3$ integrin, and it binds with higher affinity to resting than to activated platelets [30]. Figure 3 shows that echistatin and eristostatin inhibited OPG2 binding to A5 cells, to both resting and activated platelets, and to immobilized $\alpha_{IIb}\beta_3$. In all cases, eristostatin was a stronger inhibitor of OPG2 binding than echistatin. Eristostatin inhibited OPG2 binding to resting and activated platelets with IC_{50} values of 5.6 and 10.5 nM respectively. The corresponding IC_{50} values for echistatin were 35.7 and 23.7 nM respectively. Similar results were obtained for monoclonal antibody AP7, which is a mutated form of OPG2 (results not shown).

Inhibitory effect of disintegrins on binding of A2A9 to $\alpha_{IIb}\beta_3$ integrin

Monoclonal antibody A2A9 binds to A5 cells, to platelets and to immobilized $\alpha_{IIb}\beta_3$ (Figure 4) but it does not bind to VNRC3 cells or to immobilized $\alpha_v\beta_3$ (results not shown). Eristostatin strongly inhibited the binding of A2A9 to resting platelets (IC_{50} 9.0 nM) and ADP-activated platelets (IC_{50} 44.8 nM) (Figures 4B

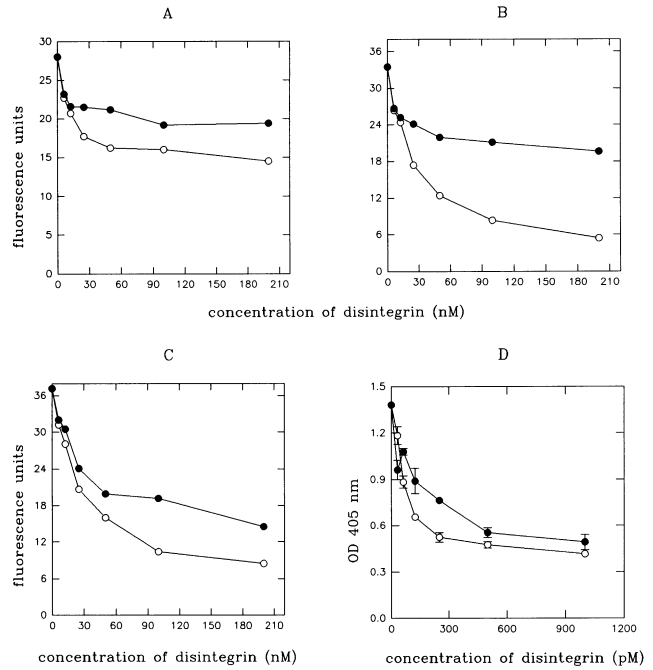

Figure 5 Effect of eristostatin (○) and echistatin (●) on binding of monoclonal antibody 7E3 to $\alpha_{IIb}\beta_3$ integrin

Flow cytometry using A5 cells (**A**), resting platelets (**B**) and ADP-activated platelets (**C**) and and ELISA assay (**D**) with immobilized $\alpha_{IIb}\beta_3$ were performed as described in the caption to Figure 3 except that 7E3 was substituted for OPG2.

Table 3 Inhibitory effect of eristostatin and echistatin on the binding of monoclonal antibody 7E3 to resting platelets in the presence of saturating concentrations of each disintegrin

The resting platelets were first incubated with 2 μM of eristostatin or echistatin for 15 min at room temperature, followed by incubation with 100 nM (or 200 nM) eristostatin or 100 nM (or 200 nM) echistatin under the same conditions. Binding of 7E3 monoclonal antibody to platelets preincubated with disintegrins was analysed by flow cytometry as described in the Materials and methods section. The values are means ± S.D. for three experiments and correspond to fluorescence units. The mean fluorescence units of platelets treated with 7E3 (without disintegrins) is 38.4. $P =$ 0.11 and 0.20 comparing 2 μM eristostatin alone and 2 μM eristostatin with 100 and 200 nM eristostatin respectively; $P = 0.0053$ and 0.0085 comparing 2 μM eristostatin alone and 2 μM eristostatin with 100 and 200 nM echistatin respectively; $P = 0.00018$ and 0.000033 comparing 2 μM echistatin alone and 2 μM echistatin with 100 and 200 nM eristostatin respectively; $P = 0.55$ and 0.19 comparing 2 μM echistatin alone and 2 μM echistatin with 100 and 200 nM echistatin respectively.

First incubation	Second incubation, additive				
Disintegrin	Buffer	+100 nM eristostatin	+200 nM eristostatin	+100 nM echistatin	+200 nM echistatin
2 μM eristostatin	3.25 ± 0.11	3.08 ± 0.09	3.13 ± 0.08	2.49 ± 0.21	2.49 ± 0.25
2 μM echistatin	10.44 ± 0.33	7.13 ± 0.28	4.44 ± 0.38	10.59 ± 0.21	9.91 ± 0.45

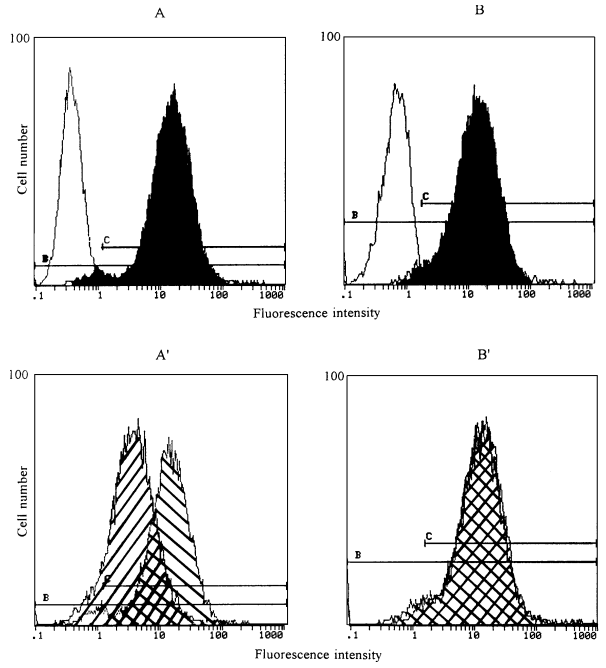

Figure 6 Effect of eristostatin and echistatin on binding of monoclonal antibodies 7E3 and LM609 to VNRC3 cells

VNRC3 cells were incubated with 7E3 (1 μg per sample) (**A**, **A′**) or LM 609 (1 μg per sample) (**B**, **B′**) after exposure to either 200 nM eristostatin (▨) or 200 nM echistatin (▨). Cells were then incubated with FITC-conjugated goat anti-mouse IgG, fixed and analysed by flow cytometry as described in the Materials and methods section. In parallel, binding of monoclonal antibodies alone to VNRC3 cells (■) and control binding of monoclonal antibodies to untransfected CHO-K1 (□) were determined.

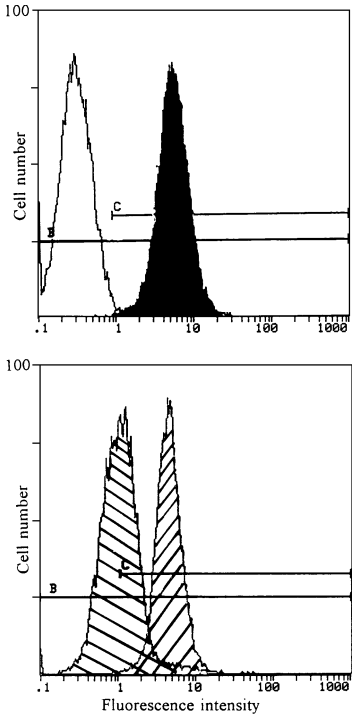

Figure 7 Effect of 7E3 and LM 609 monoclonal antibodies on binding of FITC-echistatin to VNRC3 cells

VNRC3 cells (5×10^5 per sample) were incubated with 1 μg of 7E3 or LM 609 at 4 °C for 45 min. After washing, 2 μM FITC–echistatin (or 1 μg of FITC–BSA as control) was added for 15 min at room temperature, and the excess FITC-labelled material was removed by washing three times. After fixation, cells were analysed by flow cytometry. ■, Binding of FITC–echistatin; ▨, binding of FITC–echistatin + 7E3; ▨, binding of FITC–echistatin + LM 609; □, binding of FITC–BSA.

and 4C) but it had only a small effect on A2A9 binding to A5 cells (Figure 4A). Echistatin had a very small inhibitory effect on the binding of this antibody to either platelets or A5 cells. Both eristostatin and echistatin inhibited binding of A2A9 to purified $\alpha_{IIb}\beta_3$ in a similar dose-dependent manner (Figure 4D) with IC_{50} values of 26.4 pM for eristostatin and 38.1 pM for echistatin.

Inhibitory effect of disintegrins on binding 7E3 to $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ integrins

Antibody 7E3 is an $\alpha_{IIb}\beta_3$ complex-dependent monoclonal antibody that binds with a faster rate to activated than to resting platelets [33]. This antibody is also known to recognize $\alpha_v\beta_3$ [43]. In our hands, 7E3 bound to resting and to activated platelets, to A5 cells and to VNRC3 cells, but it did not bind to non-transfected CHO-K1 cells. It recognized both immobilized $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ receptors. In contrast, monoclonal antibody LM 609 bound to VNRC3 cells and to immobilized $\alpha_v\beta_3$ but not to A5 cells, to platelets or to immobilized $\alpha_{IIb}\beta_3$. This antibody is known to be $\alpha_v\beta_3$ complex dependent [34].

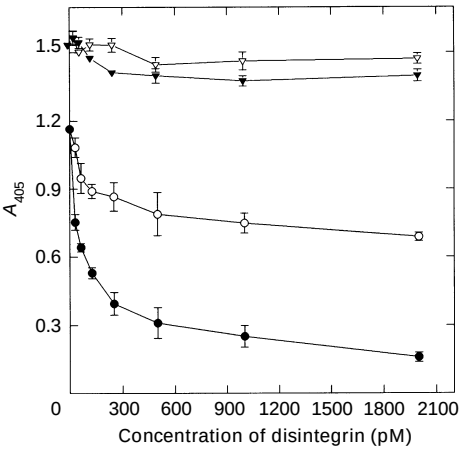

Figure 8 Effect of eristostatin and echistatin on binding of monoclonal antibodies 7E3 and LM 609 to purified $\alpha_v\beta_3$ immobilized on ELISA plates

Eristostatin (open symbols) or echistatin (filled symbols) was incubated with immobilized $\alpha_{IIb}\beta_3$ (300 ng per well) for 30 min at 37 °C. Monoclonal antibodies 7E3 (○, ●) and LM 609 (▽, ▼) (100 ng per well) were then added and the incubation was continued for 60 min at 37 °C. Binding of monoclonal antibodies was detected as described in the Materials and methods section. Error bars represent the S.D. for five independent experiments.

Figure 5 shows the effect of echistatin and eristostatin on the binding of 7E3 antibody to resting and to activated platelets, to A5 cells and to immobilized $\alpha_{IIb}\beta_3$. The pattern of inhibition of 7E3 binding by eristostatin and echistatin was similar to that reported above for the inhibition of OPG2 binding (Figure 3). The IC_{50} values for the inhibition of binding of 7E3 to resting and activated platelets by eristostatin were both similar (about 40 nM). However, echistatin inhibited binding of 7E3 to resting and activated platelets with IC_{50} values of 681 and 134 nM respectively. Table 3 shows that eristostatin has a significant ($P \ll 0.05$) inhibitory effect on 7E3 binding even in the presence of a 10–20-fold excess of echistatin. A similar but smaller inhibitory effect was observed when echistatin was added to a 10–20-fold excess of eristostatin. Addition of eristostatin or echistatin to the 10–20-fold excess of the same disintegrin caused no statistically significant changes in fluorescence units. Echistatin seemed be a strong inhibitor of 7E3 binding to VNRC3 cells, whereas eristostatin did not cause any significant inhibition. Interestingly, neither echistatin nor eristostatin inhibited the binding of LM 609 to VNRC3 cells (Figures 6A and 6A′). Accordingly, 7E3 inhibited FITC-echistatin binding to VNRC3 cells, whereas LM 609 had no effect on FITC-echistatin binding (Figure 7). This observation was confirmed by studying the effect of both disintegrins on the binding of 7E3 and LM 609 to purified $\alpha_v\beta_3$. (Figure 8). Binding of 7E3 to immobilized $\alpha_v\beta_3$ was strongly inhibited by echistatin ($IC_{50} = 97.02 \pm 14.39$) and slightly inhibited by eristostatin ($IC_{50} > 2$ nM), but neither disintegrin had any effect on the binding of LM 609.

Selective recognition of $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ by these two disintegrins was confirmed by studying the adhesion of A5 cells and VNRC3 cells to immobilized 7E3. Eristostatin and echistatin seemed to inhibit selectively the adhesion of A5 cells and VNRC3 cells respectively (Table 4). Adhesion of A5 cells to immobilized 7E3 was inhibited by eristostatin and echistatin to a similar level. However, the binding of VNRC3 cells to immobilized 7E3 was almost completely inhibited by 100 nM echistatin, whereas 200 nM eristostatin only moderately inhibited binding.

Table 4 Inhibitory effect of disintegrins on adhesion of A5 cells and VNRC3 cells to immobilized 7E3

CMFDA-labelled cells (5×10^5 per sample) were preincubated with disintegrins for 15 min at room temperature, added to immobilized 7E3 (4 μg per well) and incubated for 30 min at 37 °C. The number of adhered cells was calculated as described in the Materials and methods section. Values represent percentages of inhibition of cell adhesion for triplicate samples (means ± S.D.).

	Eristostatin		Echistatin	
Cells	100 nM	200 nM	100 nM	200 nM
A5	62.89 ± 6.32	62.37 ± 2.89	49.74 ± 7.10	57.37 ± 6.58
VNRC3	11.62 ± 9.54	38.17 ± 6.69	85.48 ± 2.07	87.55 ± 0.83

DISCUSSION

In this study we characterized echistatin and eristostatin binding sites on CHO cells transfected with $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ human integrins. Both disintegrins showed very little interaction with non-transfected CHO cells but they bound to transfected cells with a high affinity and in a reversible and saturable manner. Eristostatin and echistatin bound to 816000 sites and 495000 sites on A5 cells respectively. These numbers are somewhat higher than the number of fibrinogen receptors (150000–440000 per cell) previously reported by O'Toole et al. [36] who established this cell line. Echistatin has been reported to be a severalfold more potent inhibitor of the binding of $\alpha_v\beta_3$ to specific ligands than eristostatin, a quite selective inhibitor of $\alpha_{IIb}\beta_3$ integrin [7,44,45]. The present study shows that eristostatin and echistatin bound with similar affinity to VNRC3 cells and that the number of sites bound by eristostatin at saturation was approx. 50 % of the number of sites saturated by echistatin. However, VNRC3 cells did not adhere to immobilized eristostatin although they adhered well to immobilized echistatin. A similar observation has been reported from our laboratory [44], where we demonstrated that both purified $\alpha_v\beta_3$ and human umbilical vein endothelial cells expressing this integrin bind to immobilized echistatin but do not bind to immobilized eristostatin. All of these experiments suggest that the occupancy of the epitope to which eristostatin binds on $\alpha_v\beta_3$ does not affect cell adhesion and that echistatin and eristostatin might bind to different epitopes of the same receptors. This was also confirmed by the experiments with monoclonal antibody 7E3, which competed for echistatin but not for eristostatin binding sites on $\alpha_v\beta_3$.

We have reported previous results from flow cytometry that eristostatin and echistatin are potent LIBS inducers on platelets [46] and that echistatin but not eristostatin induces LIBS epitope on human umbilical vein endothelial cells and CHO cells transfected with $\alpha_v\beta_3$ [44]. In this study we investigated the effect of both disintegrins on the binding of ^{125}I-labelled Mab62 IgG to transfected cells and platelets. Previously, Du et al. [28] reported that Mab62 antibody binds to low-affinity sites on resting platelets and that a high concentration of the RGDS sequence results in the expression of about 26000 high-affinity sites (K_d 16 nM). In our experimental system we measured the ability of eristostatin and echistatin to induce expression of high-affinity LIBS sites on platelets or cells transfected with $\alpha_{IIb}\beta_3$ or $\alpha_v\beta_3$. In the eristostatin-induced enhancement of Mab62 binding to platelets, we found a binding affinity (K_d 15 nM) and number of binding sites (10500) similar to those reported by Du et al. [28], who studied the effect of a saturating concentration of RGDS on Mab62 binding to platelets. Mab62 bound with a much higher

affinity for transfected cells (K_d 3–5 nM) than for platelets. At disintegrin concentrations approaching saturation, the number of Mab62 high-affinity sites induced by eristostatin and echistatin on A5 cells increased 3-fold and 2-fold respectively compared with the number of sites on untreated cells. Comparison of the effect of both disintegrins on VNRC3 cells demonstrated that eristostatin caused a relatively small increase in the number of Mab62 binding sites on VNRC3 cells. VNRC3 cells treated with eristostatin failed to adhere to immobilized Mab62. In contrast, echistatin induced a similar number of binding sites on VNRC3 cells and A5 cells. In conclusion, these experiments suggest that the binding of eristostatin to the $\alpha_v\beta_3$ receptor is much less effective than the binding of echistatin with respect to the modulation of LIBS epitope. It should be noted that VNRC3 cells treated with eristostatin failed to show enhanced binding of FITC-labelled Mab62 and failed to adhere to immobilized Mab62.

We propose that the echistatin binding site is shared by $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ integrins and that the site is located close to the epitopes on $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ that are recognized by monoclonal antibody 7E3. It seems that the echistatin binding site on $\alpha_v\beta_3$ is not recognized by monoclonal antibody LM 609 (Figure 7), a well characterized monoclonal antibody that recognizes this heterodimer [34]. We propose that eristostatin and echistatin bind to different, although overlapping, epitopes on $\alpha_{IIb}\beta_3$ integrin. With transfected cells and platelets, eristostatin was a much more potent inhibitor than echistatin of the binding of three monoclonal antibodies, OPG2, A2A9 and 7E3 (Figures 3–5). However, the difference between echistatin and eristostatin was very small when their ability to inhibit the binding of OPG2, A2A9 and 7E3 to isolated receptors (Figures 3D, 4D and 5D) was compared. This may be due to conformational changes occurring during the isolation of the receptor and its immobilization. In all cases, disintegrins more strongly inhibited the binding of monoclonal antibodies to platelets than to transfected cells. This was particularly pronounced in experiments with A2A9 (Figure 4). Interestingly, the binding of this antibody to receptors on platelets and A5 cells was slightly inhibited by echistatin but was significantly inhibited by eristostatin. It seems that the binding site of eristostatin on $\alpha_{IIb}\beta_3$ and the A2A9 epitope may be located close to each other.

The strongest inhibitory effect of eristostatin was observed when studying the binding of OPG2 and A2A9 to resting gel-filtered platelets (Figures 3 and 4). A decrease in this inhibitory effect in experiments with ADP-activated platelets can be explained, assuming that eristostatin binds with the same affinity to resting and to activated platelets [8] whereas OPG2 and A2A9 bind with increased affinity to activated platelets [30,32]. Echistatin inhibited the binding of OPG2 to resting and to activated platelets quite well, but had little effect on the binding of A2A9 in this system. Therefore it seems that the binding sites of echistatin and A2A9 on $\alpha_{IIb}\beta_3$ integrin are completely different. Echistatin more strongly inhibited OPG2 binding to ADP-activated than to resting platelets because the binding affinity of echistatin to ADP-activated platelets doubles after activation of platelets [8]. The same results were obtained with monoclonal antibody AP7, which is a mutated form of OPG2 [31]. In AP7 the RYD sequence of wild-type OPG2 is replaced with an RGD sequence. The $Y^{104} \rightarrow G$ substitution in the Fab fragment of OPG2 to create AP7 does not cause any change in the structure of the RXG loop or in the biological activity of this antibody [31]. This was confirmed by studying the interaction of OPG2 and AP7 with eristostatin and echistatin. In contrast with A2A9 and OPG2, 7E3 bound to the same extent to resting and to activated platelets. Eristostatin had a similar inhibitory effect on binding of 7E3 to resting and ADP-activated platelets. Accordingly, echistatin exerted a greater inhibitory effect on binding of 7E3 to ADP-activated than to resting platelets, in agreement with the contention that the echistatin binding site is expressed at the higher levels after activation of platelets.

Further evidence that echistatin and eristostatin bind to different epitopes on $\alpha_{IIb}\beta_3$ is presented in Table 3. Addition of eristostatin further inhibited 7E3 binding to resting platelets even in the presence of a large excess of echistatin. A similar but less pronounced effect was observed for echistatin in the presence of a 20-fold excess of eristostatin. It can be concluded that eristostatin, echistatin and 7E3 have overlapping but different binding sites on $\alpha_{IIb}\beta_3$ integrin.

We hypothesize that the differences in the ability of echistatin and eristostatin to recognize integrin ligand binding sites reflects differences in the amino acid sequences of their RGD loops, C^{20}KRARGDDMDDYC^{32} and C^{23}RVARGDWNDDYC^{35} respectively. Our recent computer modelling studies of echistatin and eristostatin suggest that the shape of the RGD loop is influenced both by amino acid residues adjacent to the N-terminus (R^{22} in echistatin and V^{25} in eristostatin) and to the C-terminus (D^{27} in echistatin and W^{30} in eristostatin) of the RGD binding epitope [10]. It was recently also observed that amino acids closely adjacent to the RGD sequence in disintegrins are very important to their selectivity in the inhibition of platelet binding to different ligands such as fibrinogen, von Willebrand factor and fibrinectin [47]. The differences in the abilities of echistatin and eristostatin to induce LIBS epitope on $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ might result from the differences in the C-terminal amino acid sequences of these disintegrins [48].

In conclusion, our study suggests that disintegrins can be used as specific and selective probes to characterize functionally important ligand recognition sites on integrins.

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