Characterization of a monomeric disintegrin, ocellatusin, present in the venom of the Nigerian carpet viper, *Echis ocellatus*[1]

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Received 6 November 2001; revised 27 December 2001; accepted 28 December 2001

First published online 16 January 2002

Edited by Veli-Pekka Lehto

Abstract Ocellatusin is a new RGD-containing short monomeric disintegrin. It is a better inhibitor of $\alpha_5\beta_1$ integrin and a more potent inducer of the expression of a ligand-induced binding site epitope on β_1 integrin subunit than echistatin. In further contrast to echistatin, ocellatusin has a direct chemotactic stimulus on human neutrophils in vitro. The distinct effects of these two close evolutionarily related disintegrins might be explained by the presence of methionine-22 and histidine-29 in the RGD loop of ocellatusin, which are arginine and aspartic acid, respectively, in echistatin. These mutations may modulate the conformation and/or recognition properties of the integrin-binding loop of ocellatusin.

Key words: Disintegrin; Integrin antagonist; RGD peptide; Cell adhesion; Platelet aggregation

1. Introduction

Disintegrins are low molecular weight proteins present in many *Viperidae* venoms (for review see [1–3]), initially characterized as potent inhibitors of platelet aggregation via their interaction with the fibrinogen receptor, integrin $\alpha_{IIb}\beta_3$ [4]. The three-dimensional solution structures of a few disintegrins have been solved using ^{1}H nuclear magnetic resonance spectroscopy [5–8]. These investigations revealed that the major structural element responsible for the disintegrin/integrin interaction is a highly mobile loop joining two short β-strands and protruding 14–17 Å from the compact protein core. In general, the disintegrin family can be divided into two major subfamilies comprising monomeric and dimeric proteins. Dimeric disintegrins include both homo- and heterodimers linked by two disulfide bonds [9–13].

Monomeric disintegrins have been divided into three groups according to the length of the polypeptide and the number of the disulfide bonds. The first group includes the short disintegrins, which contain four disulfide bounds [14]. The second and the most numerous group comprises medium size disintegrins with six cystine linkages [3]. The third group of long disintegrins contains peptides crosslinked by seven disulfide bonds [15]. Monomeric disintegrins mostly express an RGD sequence at the apex of the integrin-binding loop and are inhibitors of the so-called RGD-dependent integrins, including the integrins $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$ and $\alpha_5\beta_1$. These three integrins play vital roles in many physiological processes such as homeostasis and thrombosis [16], angiogenesis, inflammation, bone resorption and wound healing [17], cell growth and organ development [18]. Blocking of these integrins by selective inhibitors would be of clinical relevance in the treatment of disorders such as platelet-induced thromboembolism and the progression of cancer. Monomeric disintegrins interact with their integrin target with different selectivity, and comparative experiments with native [19,20], synthetic [21] and recombinant [22,23] disintegrins have revealed that their integrin-binding characteristics depend on amino acids flanking the RGD sequence. Studies on two short disintegrins, echistatin (isolated from the venom of *Echis sochureki*) and eristostatin (from the venom of the viper *Eristocophis macmahoni*), showed that the type and position of the amino acids flanking the RGD motif determine the shape of the RGD loop [14], resulting in the specific recognition of integrin receptors by these disintegrins [19,21,22]. Eristostatin is a very potent and selective inhibitor of $\alpha_{IIb}\beta_3$, whereas echistatin potently inhibits the binding of all the three RGD-dependent integrins to their natural ligands. Conversion by recombinant techniques of the active loop of echistatin into that of eristostatin resulted in an increased potency of this hybrid disintegrin in the inhibition of $\alpha_{IIb}\beta_3$ integrin and in the loss of the inhibitory activity towards $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins [22].

Disintegrins are potent inducers of conformational changes in both subunits of integrins and lead to the expression of an LIBS (ligand-induced binding site) epitope. Truncation of the C-terminal residues of echistatin resulted in a significant decrease in its ability to induce the expression of the LIBS epi-

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[1] Dedicated to Stefan Niewiarowski, who passed away on August 25, 2001.

Abbreviations: CMFDA, 5-chloromethylfluorescein diacetate; HPLC, high-performance liquid chromatography; LIBS, ligand-induced binding site; TFA, trifluoroacetic acid; PMN, polymorphonuclear neutrophil

0014-5793/02/$22.00
PII: S0014-5793(02)02233-0

tope on both the β_3 and β_1 integrin subunits [22,23], strongly indicating that the potency of LIBS epitope induction is associated with this part of the disintegrin molecule.

Here, we report the amino acid sequence and biological characterization of the short monomeric disintegrin, ocellatusin, which was isolated from the venom of *Echis ocellatus*. This new protein possesses high homology with echistatin and eristostatin, but exhibits different potency and selectivity against the RGD-dependent integrins, which, we hypothesize, are associated with differences in the amino acid sequence of the RGD loop.

2. Materials and methods

2.1. Materials

Monoclonal antibody Mab62, provided by Dr. Mark Ginsberg (Scripps Research Institute, La Jolla, CA, USA), is specific for LIBS2, an epitope in the C-terminal region of the extracellular domain of the β_3 integrin subunit [24]. The monoclonal antibody 9EG7, which recognizes an LIBS epitope on the β_1 subunit [25], was purchased from Pharmingen (San Diego, CA, USA). Highly purified fibrinogen was a gift from Dr. A. Budzynski (Temple University Philadelphia, PA, USA). Human fibronectin and vitronectin were purchased from Sigma (St. Louis, MO, USA) and Chemicon (Temecula, CA, USA), respectively.

Cell lines: A5 cells - Chinese hamster ovary cells transfected with human $\alpha_{IIb}\beta_3$ integrin [26], were kindly provided by Dr. M. Ginsberg (Scripps Research Institute, La Jolla, CA, USA). HS.939T cells expressing $\alpha_v\beta_3$, and K562 cells expressing $\alpha_5\beta_1$ were purchased from ATCC (Manassas, VA, USA).

2.2. Purification of disintegrins

Ocellatusin and echistatin were purified from the whole venoms of *E. ocellatus* and *E. sochureki*, respectively. Lyophilized *E. sochureki* venom was purchased from Latoxan (France). A pool of venom was collected from 150 wild-caught specimens of Nigerian *E. ocellatus* snakes of all ages and of both sexes maintained in the herpetarium of the Liverpool School of Tropical Medicine. Venom was lyophilized and stored at 4°C in a dark bottle before use. The venoms were dissolved in 0.1% trifluoroacetic acid (TFA) and subjected to high-performance liquid chromatography (HPLC) on a C_{18} reverse-phase column as described [11]. Ocellatusin and echistatin were eluted with about 30% acetonitrile. Fractions containing the disintegrins were rechromatographed using the same column and developed with a 'flatter' acetonitrile gradient. The purity of the disintegrins was assessed by SDS–PAGE and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (using an Applied Biosystems DE-Pro spectrometer at the mass spectrometry facility of the Wistar Institute, University of Pennsylvania). Protein concentration was determined with the bicinchoninic acid protein quantification kit (Pierce Co.) with bovine serum albumin as a standard.

2.3. Structural characterization of ocellatusin

Purified ocellatusin was reduced and alkylated as described previously [11]. *S*-pyridylethylated (PE-) ocellatusin was initially characterized by N-terminal sequencing (using either an Applied Biosystems 477A or Beckman Porton LF-3000 instrument), amino acid analysis (using a Beckman Gold Amino Acid Analyzer after sample hydrolysis with 6 N HCl for 24 h at 110°C) and mass spectrometry (as above). For quantitation of the sulfhydryl groups, native ocellatusin (2 mg/ml in 100 mM Tris–HCl, pH 9.0, 150 mM NaCl, 6 M guanidine hydrochloride) was heat-denatured (1 min at 100° C), cooled at room temperature, and treated with iodoacetamide (10-fold molar excess over total cysteine content determined by amino acid analysis) for 1 h at room temperature. Carboxymethylcysteine was determined by amino acid analysis. The primary structure of ocellatusin was deduced from the N-terminal sequence analysis of the overlapping peptides obtained by degradation with CNBr (10 mg/ml protein and 100 mg/ml CNBr in 70% (v/v) formic acid for 6 h at room temperature, under N_2 atmosphere and in the dark). The peptides were separated by a reverse-phase HPLC using a 4×250 mm C_{18} (5 μm particle size) Lichrospher RP100 (Merck) column eluted at 1 ml/min with a linear gradient of 0.1% TFA in water (solution A) and 0.1% TFA in acetonitrile (solution B).

2.4. Cell adhesion studies

Adhesion studies of cultured cells labeled with 5-chloromethylfluorescein diacetate (CMFDA) were performed as described previously [21].

2.5. Platelet aggregation assay

Platelet aggregation assays were performed using platelet rich plas-

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                    1    5    10   15   20   25   30   35   40   45   50
Ocellatusin         DCESGPCCDNCKFLKEGTICKMA RGD NMHDYCNGKTCDCPRNPYKGEHDT
Echistatin          ECESGPCCRNCKFLKEGTICKRA RGD DMDDYCNGKTCDCPRNPHKGPAT
Eristostatin     QEEPCATGPCCRRCKFKRAGKVCRVA RGD WNDDYCTGKSCDCPRNPWNG
Multisquamatin   EGEECESGPCCRNCKFLKEGTICKRA RGD DMDDYCNGKTCDCPRNPHKGPAT
Pyramidin A         DCASGPCCRDCKFLKEGTICKRA RGD NMDDYCNGKTCDCPRNPHKGEHDP
Pyramidin B         DCASGPCCRDCKFLEEGTICNMA RGD DMDDYCNGKTCDCPRNPHKWPAP
Leucogastin A       DCASGPCCRDCKFLEEGTICNMA RGD DMDNYCNGKTCDCPRNPHKWPA
Leucogastin B       DCESGPCCRDCKFLKEGTVCRIA RGD NKHDYCNGKTCDCPSNPHKGKHH
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Fig. 1. Comparison of the amino acid sequences of the short monomeric disintegrins ocellatusin (*E. ocellatus*, this work), echistatin (*E. sochureki*) [30], eristostatin (=eristocophin) from *E. macmahoni* [29], multisquamatin (*Echis multisquamatus*) [28], pyramidin A (=echistatin β) and pyramidin B (=echistatin γ) from *Echis pyramidum leakeyi* [28,32], leucogastin A and leucogastin B from *Echis leucogaster* [28]. The cysteines are underlined and the RGD motifs are in italics. Asterisks below the multiple sequence alignment indicate residues that are absolutely conserved among this group of short monomeric disintegrins.

ma isolated from human blood in the presence of 0.4% sodium citrate as described earlier [21].

2.6. Neutrophil chemotaxis assay

The neutrophil chemotaxis assay was performed as described previously for other disintegrins [27]. Briefly, polymorphonuclear neutrophils (PMNs) isolated from the EDTA-treated peripheral venous blood obtained from healthy volunteers were suspended at a concentration of 10^6 cells/ml in RPMI (Roswell Park Memorial Institute) medium 1640 (Invitrogen Life Techn.). This suspension was added to the top wells of the Boyden chamber (containing 5 μm PVP-free polycarbonate filter) and allowed to migrate for 60 min at 37°C in a 5% CO_2 atmosphere into bottom wells containing the chemoattractants (disintegrins). In the next step, the filters were removed from the chambers, fixed, and stained using a Diff-Quick stain kit (Baxter Travenol Laboratories, Mississauga, ON, Canada). PMNs that migrated through the membrane were counted using light microscopy. Results represent the mean number of neutrophils per field obtained in three different experiments. The migration of PMNs towards RPMI medium alone was used as the negative control.

3. Results and discussion

N-terminal sequencing and mass spectrometric analysis of native ocellatusin yielded the single amino acid sequence DCESGPCCDNCKFLKEGTICKMARGDNMHDYC and an isotope-averaged molecular mass of 5598 ± 2 Da. Amino acid analysis of non-reduced, iodoacetamide-treated ocellatusin showed that it did not contain free sulfhydryl groups. After reduction and alkylation with vinylpyridine, a single peak was isolated by reverse-phase HPLC. The molecular mass of PE-ocellatusin determined by MALDI-TOF mass spectrometry was 6446 ± 1 Da. The mass difference of 848 Da clearly showed that ocellatusin contained eight cysteine residues involved in the formation of four disulfide bonds. The complete primary structure (Fig. 1) of ocellatusin was established by N-terminal sequence analysis of the native protein and HPLC-isolated CNBr-derived fragments. It contains 50 residues and its calculated isotope-averaged molecular mass (5597.2 Da) is in excellent agreement with the experimentally determined mass. The amino acid sequence of ocellatusin displays high homology with other short monomeric disintegrins isolated from *Echis* and *Eristocophis* snake venoms (Fig. 1). In particular, ocellatusin and echistatin are 80% identical. Further, during the course of our study, Okuda et al. [28] reported the amino acid sequence of ocellatin isolated from the venom of *E. ocellatus*. Ocellatin and ocellatusin are identical, except for the latter having Asp in position 30 in place of a histidine in the former, and for the presence of a C-terminal threonine in ocellatusin, which is absent in ocellatin. Although ocellatusin and ocellatin might be isoforms, the molecular mass of ocellatin, which would clarify this point, was not reported [28].

The IC_{50} for ADP-induced platelet aggregation of ocellatusin was 168 ± 20 nM, which is similar to that reported for the other short monomeric disintegrins isolated from the genus *Echis* (Fig. 1), e.g. ocellatin (104 ± 33 nM), multisquamatin (93 ± 8 nM), pyramidin A (160 ± 21 nM), pyramidin B (233 ± 48 nM), leucogastin A (360 ± 67 nM), leucogastin B (170 ± 41 nM) [28], echistatin (136 ± 29 nM). These disintegrins are significantly less active than eristostatin (IC_{50} = 59 ± 22 nM) [29], a short disintegrin isolated from the venom of *E. macmahoni*. Amino acids flanking the

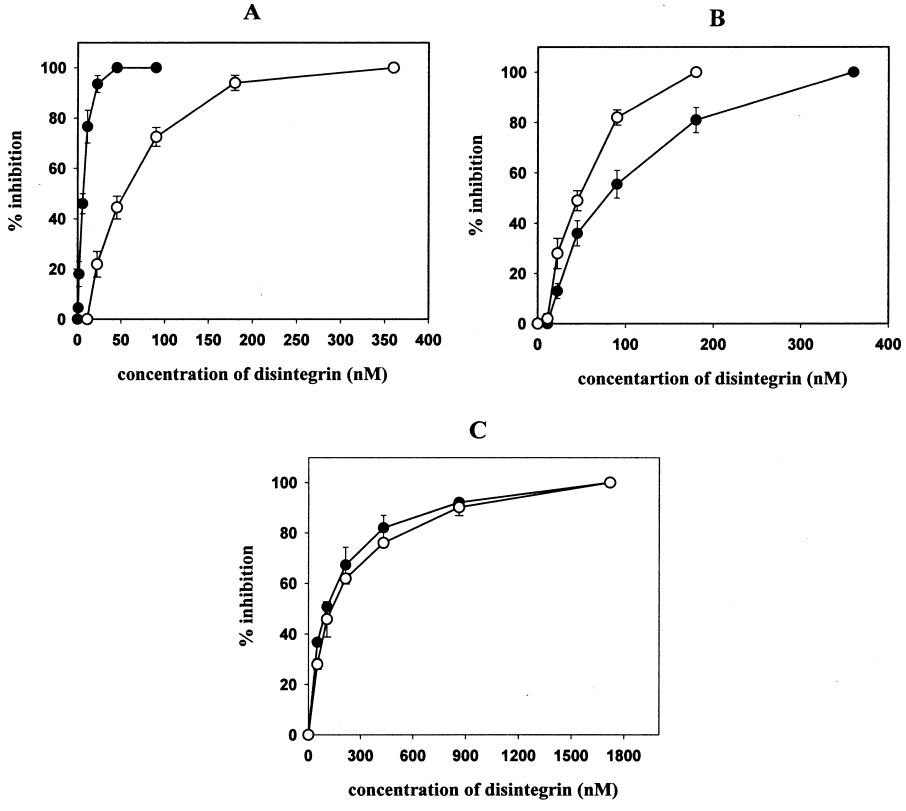

Fig. 2. Comparison of the cell adhesion inhibitory activities of ocellatusin and echistatin. The effect of ocellatusin (filled circles) and echistatin (open circles) on the adhesion of K562 cells to immobilized fibronectin (A) and A5 cells to immobilized fibrinogen (B) and HS.939T to immobilized vitronectin (C) was tested using CMFDA-labeled cells. Percentage of inhibition was calculated in relation to control sample not exposed to disintegrins. The error bars represent standard deviation from three independent experiments.

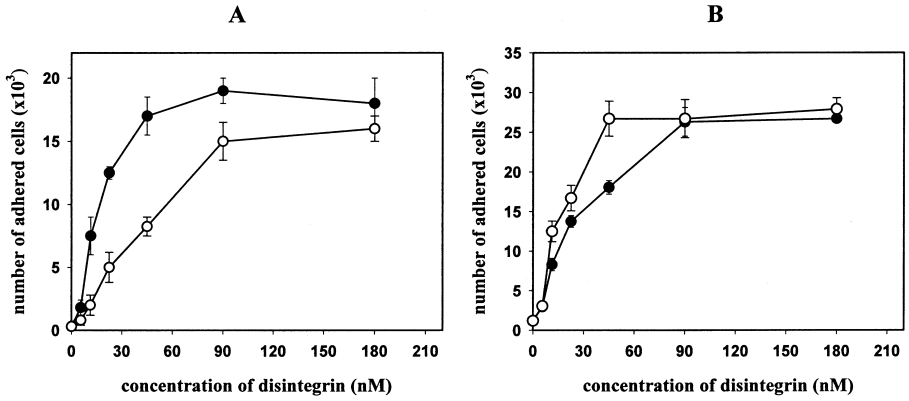

Fig. 3. Effect of ocellatusin and echistatin on LIBS expression. The ability to trigger LIBS expression was assessed for ocellatusin (filled circles) and echistatin (open circles) in an adhesion assay of K562 cells to immobilized 9EG7 monoclonal antibody (A) and A5 cells to immobilized Mab62 monoclonal antibody (B). 9EG7 recognizes LIBS epitope on the β_1 subunit of $\alpha_5\beta_1$ integrin and Mab62 binds to LIBS epitope on the β_3 subunit of $\alpha_{IIb}\beta_3$ integrin. The error bars represent standard deviation from three experiments.

RGD motif modulate the integrin inhibitory potency and selectivity of disintegrins [19–23]. Ocellatusin ($IC_{50} = 77.0 \pm 17.1$ nM) was only slightly less active than echistatin ($IC_{50} = 48.5 \pm 4.9$ nM) inhibiting the adhesion of A5 cells to immobilized fibrinogen (Fig. 2B). No significant difference in the inhibitory effect on the adhesion of HS.939T cells, a human melanoma cell line which highly expresses $\alpha_v\beta_3$ integrin, to immobilized vitronectin was observed between echistatin ($IC_{50} = 118.2 \pm 6.4$ nM) and ocellatusin ($IC_{50} = 106.2 \pm 15.2$ nM, Fig. 2C). These data suggest that differences in the amino acid sequences of the RGD loops of ocellatusin and echistatin do not affect their ability to inhibit β_3 integrins ($\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$). On the other hand, Fig. 2A shows that ocellatusin ($IC_{50} = 7.0 \pm 1.2$ nM) was over one order of magnitude more active than echistatin ($IC_{50} = 54.2 \pm 9.3$ nM) in inhibiting the adhesion of K562 cells to immobilized fibronectin mediated by integrin $\alpha_5\beta_1$.

The RGD loops of ocellatusin and echistatin [30] differ in three amino acids, which are (ocellatusin/echistatin) Met22/Arg, Asn27/Asp, and His29/Asp. The inhibitory activities towards integrin $\alpha_5\beta_1$ of a number of disintegrins containing RGDD or RGDN are indistinguishable [20,22]. On the other hand, the substitutions at positions 22 and 29 are non-conserved and may significantly affect the physicochemical properties (charge, shape) of the RGD loop and contribute to the enhanced $\alpha_5\beta_1$ inhibitory potency of ocellatusin over echistatin. However, at present it cannot be ruled out that other amino acid differences between ocellatusin and echistatin also contribute to the enhanced $\alpha_5\beta_1$ specificity of ocellatusin.

The more powerful antagonistic effect of ocellatusin compared with echistatin on the $\alpha_5\beta_1$ integrin was also reflected at the level of the expression of LIBS. Fig. 3A shows that lower concentrations of ocellatusin than echistatin were needed to induce maximal expression of the LIBS epitope recognized by the monoclonal antibody 9EG7 (anti-β_1). Noteworthy, both disintegrins induced almost equally expression of LIBS on β_3 integrin subunit recognized by the monoclonal antibody Mab62 (Fig. 3B). The ability of disintegrins to induce LIBS expression depends on both the RGD loop and the C-terminus [21,22]. Disintegrins with a truncated C-terminus or with a mutated RGD motif lack the activity to express LIBS epitopes on both β_3 and β_1 integrin subunits. The fact that ocellatusin exhibits similar to echistatin LIBS expression activity on β_3 integrins suggests that increased LIBS epitope expression on the β_1 subunit correlates with the higher affinity of ocellatusin to the $\alpha_5\beta_1$ integrin.

It is important to note that ocellatusin, but not echistatin, significantly induced chemotaxis of human neutrophils (Fig. 4) in a dose-dependent manner. Although the possibility that this effect might be linked to the interaction of ocellatusin with a neutrophil receptor that is not a target of echistatin cannot be ruled out, we favor the hypothesis that the chemotaxic activity may be related to its higher affinity for the $\alpha_5\beta_1$ integrin.

In conclusion, we report a new short monomeric disintegrin isolated from the venom of *E. ocellatus* from West Africa. Another member of this family, echistatin, was previously isolated from the venom of a snake of the same genus, *E. sochureki* [30] from a different geographical region (Afghanistan, Pakistan and India). Ocellatusin differs from echistatin in possessing (i) enhanced affinity for integrin $\alpha_5\beta_1$; (ii) higher LIBS expression on β_1; and (iii) a chemotactic effect on hu-

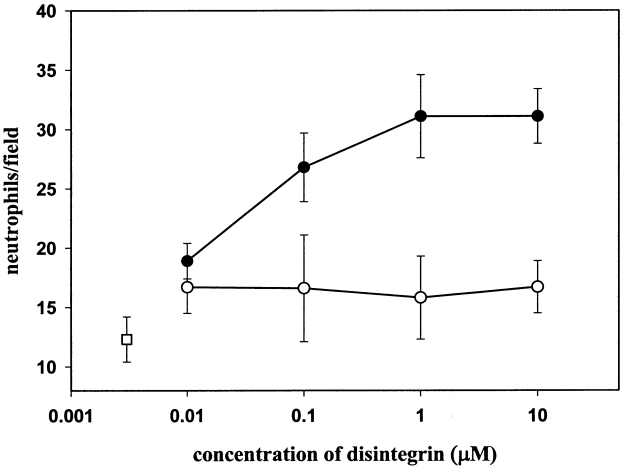

Fig. 4. Effect of ocellatusin (filled circles) and echistatin (open circles) on human neutrophil chemotaxis in vitro. Cells were allowed to migrate in the Boyden chamber towards medium alone (open squares), ocellatusin (filled circles) or echistatin (open circles). Data show mean ± standard deviation from three independent experiments.

man neutrophils. Changes in venom composition have previously been shown to reflect different prey species [31] and it may be that this is the case here. The difference in the chemotactic effects of ocellatusin and echistatin on neutrophils may suggest a different mechanism of venom action.

Acknowledgements: We thank Dr. G.D. Laing and Mr P. Rowley for venom extraction and Dr. A.S. Kamiguti for her critical reading of the manuscript. The work was supported in part by a W.W. Smith Charitable Trust Research Grant (C.M.), an American Heart Association Beginning Investigator Grant (C.M.), and by Grants PB98-0694 and BCM2001-3337 from the Ministerio de Ciencia y Tecnología (Madrid, Spain) (J.J.C.).

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