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Anti-platelet effect of cumanastatin 1, a disintegrin isolated from venom of South American *Crotalus* rattlesnake

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ABSTRACT

Disintegrins have been previously described in the venom of several snake families inhibiting signal transduction, cell-cell interactions, and cell-matrix interactions and may have therapeutic potential in heart attacks, thrombotic diseases, and cancers. This investigation describes the first disintegrin isolated from South American *Crotalus* venom (Venezuelan rattlesnake *Crotalus durissus cumanensis*), which inhibits platelet adhesion to matrix proteins. *C. d. cumanensis* crude venom was first separated on a Sephadex G-100 column into 4 fractions (SI to SIV). Crude venom and SIII fraction significantly diminished platelet adhesion to fibrinogen (Fg) and to fibronectin (Fn). Anti-adhesive SIII fraction was further separated by DEAE-Sephacel followed by C-18 reverse phase high performance liquid chromatography (HPLC). The platelet anti-adhesive fraction obtained was designated as cumanastatin-1. This disintegrin has a mass of 7.442 kDa as determined by mass spectrometry (MALDI-TOF/TOF) and pI of 8.5. Cumanastatin-1 also inhibited ADP-induced platelet aggregation with an IC_{50} of 158 nM. However, it did not significantly inhibit collagen and thrombin-induced platelet aggregation. Cumanastatin-1 considerably inhibited anti-$\alpha_{IIb}\beta_3$ integrin binding to platelets in a dose-dependent manner; however, it did not present any effect on the $\alpha_5\beta_1$ integrin or on P-selectin.

Introduction

The Venezuelan rattlesnake *Crotalus durissus cumanensis* is the most widely distributed snake in the Country and the Colombia frontier. It is mainly a savannah snake, but it has been described in piedmont, sub-tropical mountains, valleys and xerophytic regions. *Crotalus durissus cumanensis* venom shows neurotoxic, hemorrhagic, thrombin-like and fibrino(geno)lytic activities [1]. However, to date, no disintegrins have been described in this or any other South American *Crotalus* venom.

Disintegrins are peptides rich in cysteine that contain in their structure a sequence of amino acids located at the tip of a flexible hairpin loop of type RGD, KGD, MLD, KTS, ECD, VGD, MGD and WGD which recognizes integrins or cellular receptors. They are divided into five different subgroups according to their polypeptide length and number of disulfide bonds [2]. They also consist of molecules with metalloprotease activity, which are isolated from Colubridae, Elapidae and Viperidae snake venoms. Disintegrins were first isolated as short, soluble snake venom platelet aggregation inhibitors [3]. They are effective and specific antagonists of several integrins, which are a family of transmembrane cell surface proteins that intervene on cell-cell interactions and the adhesion of cells to extracellular matrix proteins and other ligands. Integrins are heterodimeric structures composed of the association of α and β subunits [4]. In humans there are at least 15 different α subunits and 8 different β subunits, and they can combine to form proteins with diverse ligand specificities and biological activities. Integrins characterize the main class of adhesion receptors and contribute to the preservation of tissue integrity [5]. Initially it was thought that snake venom disintegrins were only responsible for the inhibition of platelet aggregation via the $\alpha_{IIb}\beta_3$ integrin found on the surface of platelets. However, now it is very well known that disintegrins bind to many types of cell lines including, endothelial, inflammatory and cancer cells, which may affect important cell functions such as wound healing, development, extravasations of lymphocytes, and tumor biology [6–9].

In addition to their potent antiplatelet activity, disintegrins revealed new uses in the diagnosis of cardiovascular diseases. The purpose of this study was to isolate and characterize a disintegrin which could be used as a tool for investigating cell-matrix and cell-cell interactions in several pathological processes in which platelets are involved such as thrombosis, cancers, inflammation, autoimmune diseases and viral infections. No study has been previously reported on the purification and characterization of disintegrins from South American *Crotalus* venom. This work characterizes the first disintegrin, purified from venom of a Venezuelan rattlesnake, *C. d. cumanensis*, capable of

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inhibiting platelet adhesion to fibronectin and fibrinogen and also inhibiting ADP-induced platelet aggregation.

Materials and methods

Reagents

Chromogenic substrates were obtained from Chromogenix AB (Milano, Italy). Human fibrinogen (10% w/w of plasminogen as contaminant), bovine alpha thrombin, single-chain t-PA (sct-PA), two chains u-PA (tcu-PA) and plasmin were obtained from American Diagnostica Inc. (Greenwich, CT, USA). Sephadex G-100 from Pharmacia (Uppsala, Sweden). Trifluoroacetic acid was obtained from Riedel-de Haën (Germany). ADP, collagen and thrombin as platelet aggregation inducer was purchase from Chrono-log (Havertown, USA). Anti P-Selectin-FITC, anti-GII_b/III_a-PE (CD41/CD61) and anti-$\alpha_5\beta_{1,}$ secondary antibody conjugated with FITC and non-specific immunoglobulin G (IgG) were purchase from Becton Dickinson (FACStar, Braintree, MA, U.S.A.). Echistatin, bicinchoninic acid, *p*-nitrophenyl phosphate, iodoacetamide, ethylene glycol-bis-N,N,N',N'-tetraacetic acid (EGTA), apyrase, prostaglandin E1, aprotinin, ethylenediaminetetraacetic acid (EDTA), soybean trypsin inhibitor (SBTI), phenylmethylsulfonyl fluoride (PMSF), 1-10 phenantroline, benzamidine/HCl, trypan blue, DEAE-Sephacel and other reagents used in this study were from Sigma Chemical Co (St. Louis, MO, USA).

Snakes and venom

Crotalus durissus cumanensis venoms from 6 adult snakes were obtained from San Lorenzo, Anzoátegui state, Venezuela. They were housed at the Serpentarium of the Instituto de Medicina Tropical, Universidad Central de Venezuela, Caracas, Venezuela. Venom pools were filtered through a 0.45-μm membrane, lyophilized, divided into 30 mg samples and stored at -80 °C.

Protein concentration determination

The protein concentration was determined by the Lowry et al. [10] method and by Bicinchoninic method [11,12].

SDS-PAGE analysis

Polyacrylamide gel electrophoresis was done by the Tris-Tricine-system method [13], using a Mini-Protean II system (Bio-Rad Laboratories, Hercules, CA).

Isoelectric focusing

Isoelectric focusing (IEF) was done using a 5% polyacrylamide gel with a pH gradient from 3 to 10, following the Garfin method [14]. Standard proteins were used as a reference to determine the isoelectric point (pI) range. The gels were stained with Coomassie Blue-0.5% $CuSO_4$.

Fibrinolytic activity

Fibrinolytic activity of crude venom and fractions was studied by the fibrin plate method as proposed by Marsh and Arocha-Piñango [15]. Briefly, fibrin plates were prepared using 3-cm diameter Petri dishes: 1.5 mL of a 0.1% purified fibrinogen (containing 10% plasminogen as contaminant) in imidazol saline buffer, pH 7.4 was clotted by adding 75 μL bovine thrombin (10 IU/mL, in 0.025 M $CaCl_2$). The mixture was incubated at room temperature for 30 min. Then, a 10 μL sample was put on the fibrin film, and after 24 h incubation at 37 °C the diameters of the lysed areas were measured. Fibrinolytic activity was expressed as the diameter of the lysis area per μg of protein ($mm^2/\mu g$). Human plasmin, sct-PA and tcu-PA were used as positive controls.

Amidolytic activity

Amidolytic activity of crude venoms and fractions was estimated by a micromethod standardized in our laboratory [16]. Briefly, in 96 wells polystyrene plates a mixture of 80 μL of the recommended buffer for each substrate, 10 μL of the venom sample and 10 μL of chromogenic substrate were placed in each well. The final concentrations for the substrates were 3.5 mM S-2251, 1.2 mM S-2288, 1 mM S-2302, and 0.16 mM S-2444 in which similar activity to plasmin, t-PA, kallikrein and urokinase were determined, respectively. After incubation at 37 °C for 5 or 15 min, the absorbance at 405 nm was measured. One unit of amidolytic activity was expressed as mUA 405 nm/min. Specific activity was calculated as mUA/min/μg.

Crotalus durissus cumanensis venom fractionation

Anti-adhesive component was achieved by three chromatographic steps. First, samples from *C. d. cumanensis* venom were run in a preparative procedure using molecular exclusion chromatography on a Sephadex G-100 column equilibrated at 4 °C with 50 mM ammonium acetate buffer pH 6.8. Venom samples (250 mg/ 5 mL) were dissolved in the equilibrating buffer and injected into the column. The elution was carried out with the same buffer at 0.2 mL/min flow rate and monitored at 280 nm [17]. Secondly, the eluted fractions with platelet anti-adherent activity from Sephadex G-100 column were re-chromatographed on an anionic exchange DEAE-Sephacel column, equilibrated at 4 °C with 10 mM Tris-HCl, pH 8.6. The proteins were eluted at a 0.5 mL/min flow rate with NaCl gradient from 0 to 1 M in the equilibrium buffer. The eluted material was monitored at 280 nm. Finally, the active fractions were pooled, dialyzed against 0.1 % (v/v) trifluoroacetic acid in water (TFA) and centrifuged for 5 min at 5000 × *g* to remove the insoluble proteins. The pellet was discarded, and the supernatant was applied to a reverse phase Bio-Sil C-18 HL 90-5 S column (5 μm; 4.6×150 mm-, Bio-Rad, Hercules, CA) that was equilibrated with 0.1 % TFA, at a 1 mL/min flow rate, using a Waters 1525 binary HPLC pump (Milford, MI, USA). The column was eluted at room temperature with an acetonitrile linear gradient of 0–80% (v/v) in 0.1% TFA over 60 min. A Waters 2487 dual λ absorbance detector (Milford, MI, USA) was used to monitor absorbance at 215 nm and Waters Breeze software was used to control the HPLC system and store the data. The fraction displaying platelet anti-adherent activity was lyophilized. This component was named cumanastatin-1.

Mass spectrometry analysis (MALDI-TOF-TOF)

Cumanastatin-1 was resuspended in 10 μL of 50% acetonitrile/50%-0.1% triflouracetic acid in 18 mega ohm water and desalted using a C-18 ZipTip (MILLIPORE, Bedford, MA). One microliter of sample was combined with 1 μL sDHB matrix (10 mg/mL) and 1 μL of the mixture was spotted onto the MALDI plate. Mass analysis was performed with Flex Control software on the AUTOFLEX II-TOF/TOF Mass spectrometer (Bruker Daltonics) in positive reflectron mode using external standards (Bruker Protein Calibration Standard I, Part #206355) in order to determine the monoisotopic mass of the intact protein.

Preparation of platelet-rich plasma and washed human platelet suspension

Blood freshly obtained from healthy volunteer adults who had confirmed not using any drugs known to interfere with platelet function, during the previous 14 days, was collected with an acid citrate/dextrose solution-ACD (85 mM sodium citrate; 71 mM citric acid; 111 mM dextrose) at 6:1 ratio, after informed consent (Ethics Committee approval, Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela). Platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at 190 × *g* for 20 min. Then, it was centrifuged at 1700 × *g* for 15 min at 24 °C, in presence of 0.25 μg/mL prostaglandin E1 (platelet activation inhibitor). Then, the platelet