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Short Communication

Cloning and Characterization of Adinbitor, a Novel Disintegrin from the Snake Venom of *Agkistrodon halys brevicaudus stejneger*

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Abstract Adinbitor was cloned from *Agkistrodon halys brevicaudus stejneger* and characterized as a novel disintegrin. In this study, total RNA was extracted from venom gland and used in RT-PCR to generate a cDNA which is 219 bp long. The sequence encoded a polypeptide composed of 73 amino acids, including 12 cysteines, an RGD motif, and the signature motif of disintergrin. Recombinant Adinbitor (rAdinbitor) was expressed in *E. coli* and purified by using the His•Bind affinity chromatography. The IC_{50} for inhibiting human platelet aggregation and bFGF-induced proliferation of ECV304 cells was 6 μM and 0.89 μM respectively. Furthermore, Adinbitor significantly inhibited angiogenesis both *in vivo* and *in vitro*. Taken together, these results suggested that Adinbitor had typical functions of disintegrins.

Key words cloning; Adinbitor; disintegrin; platelet aggregation; angiogenesis

Disintegrin is a family of small proteins mainly derived from snake venoms. Most of the disintegrins contain RGD or KGD sequence which is the structural motif recognized by the platelet fibrinogen receptor $\alpha_{2b}\beta_3$, and they also act as potent antagonists of several integrins including $\alpha_v\beta_3$ and $\alpha_5\beta_1$ which are expressed on vascular endothelial cells and some tumor cells. In addition to disintegrins' potent antiplatelet activity, studies on disintegrins have revealed their new applications in inhibiting angiogenesis and tumor growth [1–15].

To find a novel disintegrin from Chinese snake (*Agkistrodon halys brevicaudus stejneger*) and study its functions of antithrombotic and anti-tumor, gene cloning method was used. We reported here that Adinbitor was a novel disintegrin from Chinese snake. We demonstrated cDNA cloning and expression of Adinbitor, and characteristic of its biological activities.

Materials and Methods

Materials

Chinese snake (*Agkistrodon halys brevicaudus stejneger*) was obtained from Lushun of Liaoning, China. Chick embryos were from Dalian Hanwei Company. Human umbilical vein endothelial cells line, ECV304, was purchased from Wuhan University (Hubei, China). Trizol reagent, M199, and MTT were purchased from Gibco BRL. RT-PCR kit, *Nde*I, and *Hin*dIII were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). pET23b, DH5α, and BL21 were conserved in our lab. His•Bind Column was purchased from Novagen. Matrigel was purchased from Becton Dickinson. Basic fibroblast growth factor (bFGF) was purchased from Peprotech Ec Ltd.. Anti-$\alpha_v\beta_3$ antibody was purchased from Chemicon. Hoechst apoptosis kit was purchased from Beyotime Biotechnology.

cDNA cloning of Adinbitor

Total RNA was extracted from venom gland using Trizol reagent and used for RT-PCR. According to the homo-logous protein sequence of disintegrins published on NCBI, we deduced the DNA sequences and designed two oligonucleotide primers: primer 1, 5′-TTATG-CATATGGAGGCCGGAGAAGAATG-3′ (*Nde*I restriction site was underlined); primer 2: 5′-TTATGAAGCTTG-GCATGGAAGGGATTTC-3′ (*Hin*dIII restriction site was underlined). The primers synthesizing and DNA sequen-

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cing were provided by TaKaRa Biotechnology Co., Ltd. (Dalian).

Construction of expression vector

To generate fusion protein with C-terminal His-tag, the PCR products digested with *Nde*I and *Hin*dIII were ligated into pET23b vector. The recombinant construct, pET23b-ab, was then transformed into the *E. coli* BL21.

Expression and purification of Adinbitor

E. coli BL21 with pET23b-ab was grown at 37 °C in LB medium, and IPTG (final concentration 1 mM) was added to induce recombinant protein (rAdinbitor) expression. According to specificity of His•Bind column, cells were harvested and resuspended in ice-cold binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and sonicated on ice. After centrifuged the lysate at 14,000 *g* for 20 min, supernatant filtrated through a 0.45 μm membrane was incubated with Ni-NTA resin and bound fusion protein was eluted with increasing amount of imidizole. The purified protein was resolved on 16.5% Tricine SDS-PAGE [16]. The protein concentration was determined by Lowry method [17].

Platelet aggregation assay

Platelet aggregation assays were performed in human platelet as described previously [1–3]. Platelet was concentrated by centrifuging at 800 *g* for 5 min at room temperature and the platelet-rich plasma (PRP) was decanted and saved. Platelet-poor plasma (PPP) was prepared from the remaining platelet solution by further centrifuging at 4000 g for 10 min. PRP was diluted to 3×10^5 platelets per microliter with PPP. The diluted PRP (300 μl) plus 30 μl of either the rAdinbitor in PBS or PBS alone was incubated in an aggregometer (LBY-NJ2) for 3 min at 37 °C. Light transmittance was recorded and ADP (final concentration 3 μM) was added to initiate platelet aggregation. Inhibition of platelet aggregation was measured at the maximum aggregation response.

Inhibition of ECV304 proliferation and viability assay

ECV304 were plated onto 96-well culture plates and maintained with bFGF (3 ng/ml) in M199 at 37 °C in 5% CO_2 for 24 h, then the cells were treated with rAdinbitor at various concentrations for 24 h. Cells were incubated with MTT at a final concentration of 0.5 mg/ml for 4 h. After lysis in dimethyl sulfoxide (DMSO), the absorbance of cell lysate at 490 nm was measured.

Apoptosis analysis was performed by using Hoechst apoptosis kit. ECV304 cells (1×10^6 cells/ml) were added onto cover slip placed in a 6-well plate. After 24 h, various amounts of rAdinbitor were added to induce apoptosis for 24 h. Cell fixation and staining procedures were identical to the kit descriptions. Nuclear morphology was examined by fluorescence microscopy. Individual nucleus was visualized at 100-fold magnification to distinguish the normal uniform nuclear pattern from the characteristic condensed chromatin pattern of apoptotic cells.

ECV304 tube formation assay

According to the product specification, Matrigel was diluted to 2.5 mg/ml in the presence or absence of bFGF (30 ng/ml) and added to 6-well plate in a total volume of 1 ml in each well to form the gel layer. Then ECV304 cells (1×10^6 cells/ml) were seeded onto Matrigel. After incubation with or without rAdinbitor for 24 h, ECV304 cells were fixed in 4% paraformaldehyde and photographed under the Olympus inverted contrast phase microscope.

Chick CAM angiogenesis assay

Egg of 6-day-old chick embryo was opened with a 1.0 cm^2-window. According to the method described previously [4–6], filter paper disk saturated with bFGF (200 ng/disk) in the presence or absence of test peptide or an equal volume of PBS (final in 20 μl) was applied to the top of the chicken chorioallantoic membrane (CAM). After 48 h incubation, CAM was photographed by digital camera.

Results and Discussion

Cloning, expression and purification of Adinbitor

Sequence analysis showed that the cDNA of Adinbitor was 219 bp long and the deduced amino acid sequence contained 73 amino acids including 12 cysteines as well as the motif-RGD (Fig. 1). NCBI Blasting results revealed that Adinbitor was highly homologous to other disintegrins: 91.78% homology with saxatilin [1], 79.45% homology with barbourin [2], 63.64% homology with bitistatin [3] region corresponding to amino acid 11 to 84, 63.01% with salmonsin3 [7] region corresponding to amino acid 74 to 147.

Fusion rAdinbitor with the C-terminal His-tag is a 9 kD soluble protein. It was purified by using the His•Bind column and resolved on Tricine SDS-PAGE. The purified Adinbitor migrated as a single band (Fig. 2).

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  1  GAG GCC GGA GAA GAA TGT GAC TGT GGC TCT CCT GGA AAT CCG TGC TGT GAT GCT GCA ACC
  1   E   A   G   E   E   C   D   C   G   S   P   G   N   P   C   C   D   A   A   T

 61  TGT AAA CTG AGA CAA GGA GCA CAG TGT GCA GAA GGA CTG TGT TGT GAC CAG TGC AGA TTT
 21   C   K   L   R   Q   G   A   Q   C   A   E   G   L   C   C   D   Q   C   R   F

121  ATG AAA AAA GGA ACA GTA TGC CGG ATA GCA AGG GGT GAT GAC ATG GAT GAT TAC TGC AAT
 41   M   K   K   G   T   V   C   R   I   A   R   G   D   D   M   D   D   Y   C   N

181  GGC ATA TCT GCT GGC TGT CCC AGA AAT CCC TTC CAT GCC
 61   G   I   S   A   G   C   P   R   N   P   F   H   A
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Fig. 1 The cDNA sequence and deduced amino acid sequence of Adinbitor

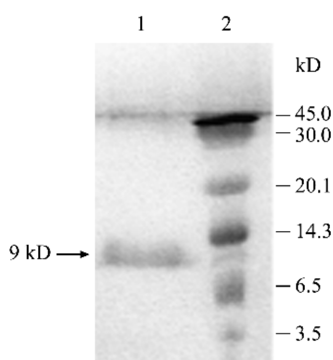

Fig. 2 Tricine SDS-PAGE of the homogeneous protein purified by affinity chromatography

1, the purified fusion protein; 2, Rainbow™ coloured low molecular weight marker.

Platelet aggregation assay

Dose-dependent inhibition of human platelet aggregation by rAdinbitor was measured in ADP-induced platelet aggregation assay system. Human platelet aggregation was completely inhibited by rAdinbitor at concentration of 8.4 μM. The IC_{50} value of rAdinbitor inhibiting platelet aggregation was 6 μM (Fig. 3).

According to the report by Hong *et al.* [1], IC_{50} value of saxatilin, salmonsin, and synthetic peptide GRGDSP for inhibiting human platelet aggregation was 127 nM, 131 nM, and 270 μM respectively. These indicated that rAdinbitor was 46-fold less active than saxatilin and salmonsin and 45-fold more active than the synthetic peptide in human platelet aggregation assay.

ECV304 proliferation and viability assay

To examine the ability of rAdinbitor of inhibiting angiogenesis, we used ECV304 cell proliferation and viability assay system. rAdinbitor was able to inhibit bFGF-induced proliferation of ECV304 in a dose-dependent manner. The IC_{50} value of rAdinbitor was 8 μg/ml (0.89 μM), while saxatilin was 1.12 μM [1] and rhodostomin was about 0.27 μM [4]. These indicated that rAdinbitor is much more similar to saxatilin due to their high homology. Further experimental investigation revealed that anti-$\alpha_v\beta_3$ antibody also significantly inhibited the cell proliferation induced by bFGF (Fig. 4). Studies on rhodostomin have confirmed that angiogenesis elicited by bFGF could be inhibited by a selective $\alpha_v\beta_3$ blockade of endothelial cells [4], thus it is possible to postulate that rAdinbitor also binds to integrin $\alpha_v\beta_3$, which is closely associated with bFGF-

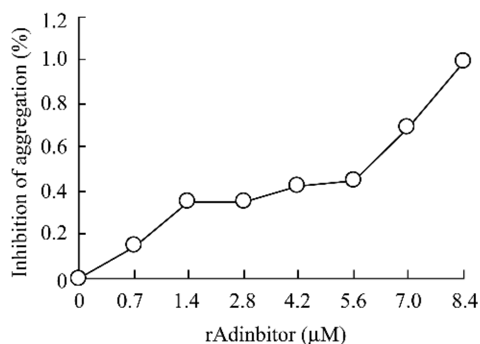

Fig. 3 Dose-dependent inhibition of human platelet aggregation by rAdinbitor

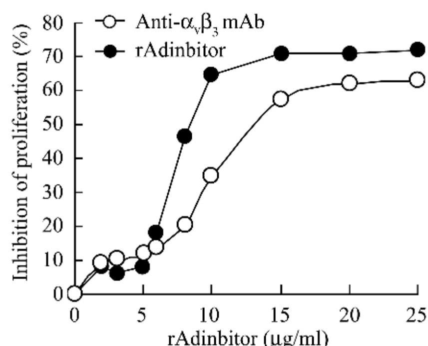

Fig. 4 Inhibition on ECV304 cells proliferation

induced ECV304 proliferation.

To investigate the effect of rAdinbitor on apoptosis, rAdinbitor treated or untreated ECV304 cells were stained with Hoechst 33258, a fluorescent DNA-binding dye, to identify the apoptotic nuclei. As shown in Fig. 5, the nuclei of the cells treated with rAdinbitor were stained much brighter than that of untreated cells due to chromatin condensation. This result demonstrated that Adinbitor could induce apoptosis in ECV304 cells, which is one of the biological functions for disintergrin [4,5,8,9].

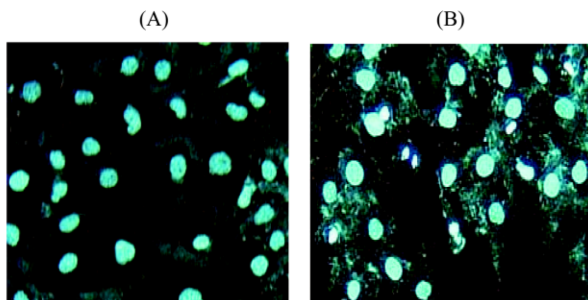

Fig. 5 rAdinbitor-induced nuclear morphological changes in ECV304

ECV304 nuclei were examined by using Olympus fluorescence microscopy in the absence (A) and presence (B) of 2.22 μM rAdinbitor for 24 h (100×). In (B), rAdinbitor-induced condensed, coalesced, and segmented nuclei were shown.

Effect of Adinbitor on angiogenesis *in vivo* and *in vitro* elicited by bFGF

To study the effect of rAdinbitor on bFGF-induced angiogenesis, we used *in vivo* and *in vitro* assay systems.

CAM assay was performed *in vivo*. Results showed that, CAMs treated with PBS were well vascularized [Fig. 6(A)], whereas CAMs treated with rAdinbitor generated discontinuous blood vessels [Fig. 6(B)]. Therefore, rAdinbitor displayed a significant inhibition on bFGF-induced angiogenesis *in vivo*. Similar results have already been reported for other disintegrins [4–6].

Furthermore, *in vitro* angiogenesis assays were performed by using three-dimensional gel consisting of diluted Matrigel. As shown in Fig. 7(A), when ECV304 cells were seeded on diluted Matrigel without the addition of bFGF, they showed only a few spontaneous tube formations. On the other hand, when ECV304 cells were seeded on the diluted Matrigel with addition of bFGF (30 ng/ml), cells displayed high motility and cell-cell communication, and aligned and formed an anastomosed capillary-like network within 16 h [Fig. 7(B)]. However, bFGF-induced tube formation is significantly inhibited by adding rAdinbitor [Fig. 7(C)]. Tube formation *in vitro* affected by rhodostomin or accutin gave the same results [4,5].

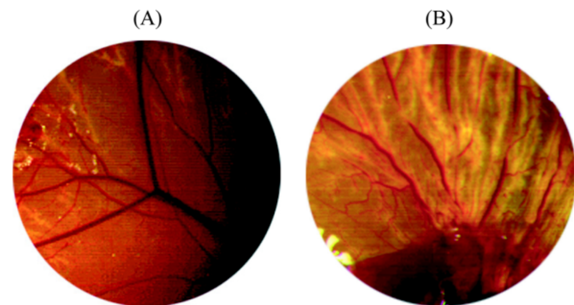

Fig. 6 Effect of rAdinbitor on bFGF (200 ng/disk) induced CAM angiogenesis

Methylcellulose disk containing PBS (A) or 50 μg Adinbitor (B) was implanted on each CAM from ten 6-day-old chick embryos. CAM tissues were photographed by digital camera (5×).

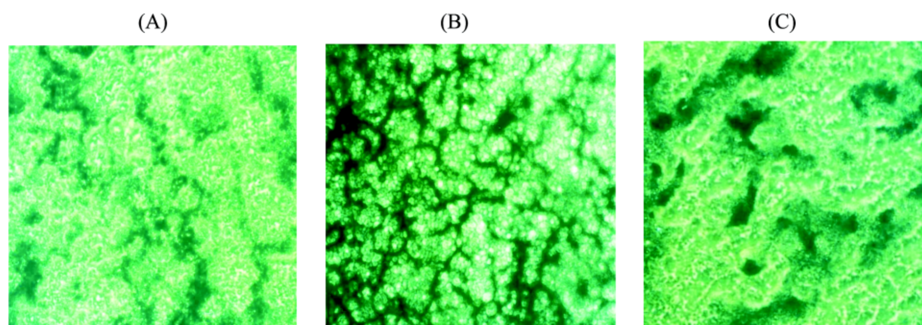

Fig. 7 rAdinbitor inhibited bFGF-induced ECV304 tube formation in diluted Matrigel

(A) ECV304 cells (1×10^6 cells/ml) were seeded on diluted Matrigel without bFGF. (B) ECV304 cells were seeded on diluted Matrigel in presence of bFGF (30 ng/ml) for 24, and then treated by PBS. (C) ECV304 cells were seeded on diluted Matrigel in presence of bFGF (30 ng/ml) for 24, and then treated by rAdinbitor (2 μM). Pictures were taken under Olympus inverted contrast phase microscope (100×).

These results clearly demonstrated that rAdinbitor could inhibit bFGF-elicited angiogenesis *in vivo* and *in vitro*. Because growth and progression of primary solid tumors are highly dependent on angiogenesis and an avascular tumor rarely grows to a size larger than 2 to 3 mm [9–15]. rAdinbitor would be useful in anti-tumor study.

Disintegrin, a component of some snake venoms, has been confirmed to possess two typical functions: inhibition of platelet aggregation and angiogenesis. In this work, rAdinbitor was obtained by gene engineering and confirmed to have the two typical functions of disintegrin. This will be valuable in developing antithrombotic and anti-tumor agents.

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