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Short communication

Characterization of a pro-angiogenic, novel peptide from Russell's viper (*Daboia russelii russelii*) venom

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ABSTRACT

Present report shows for the first time on the induction of *in vitro* angiogenesis by a 3.9 kDa novel peptide (RVVAP) purified from Russell's viper venom. Secondary structure of RVVAP is made up of 36.8% α-helix, 33.3% β pleated sheets and 29.9% turns. Optimum angiogenesis and significant elevation in endothelial migration were observed at 50 ng/ml of RVVAP treatment; above this concentration, progressive decrease in wound healing was noted. RVVAP (1.0 μg/ml) was non-cytotoxic to U87-MG, HeLa and HT-29 cells; however, increasing the RVVAP concentration above 500 ng/ml resulted in induction of chromosomal aberrations and delay in cell cycle kinetics of Chinese hamster ovary cells.

1. Introduction

Angiogenesis involves differentiation of pre-existing vascular network to form new blood vessels. This is a natural biological phenomenon occurring during embryonic development, ovulation and wound repair (see reviews Goodwin, 2007; Otrock et al., 2007). This process is also associated with development of numerous pathological conditions such as cancer, arthritis and diabetic retinopathy (Goodwin, 2007; Otrock et al., 2007). In the absence of angiogenesis, tumor growth is restricted to a microscopic size and tumor cells do not enter into the circulation to initiate the process of metastasis (Otrock et al., 2007). In view of physiological and therapeutic significance of angiogenesis (Folkman, 1995), remarkable progress has been made in recent year to characterize several pro-angiogenic factors as well as to understand their molecular mechanism(s) of action both in *ex-vivo* and *in vivo* conditions.

Russell's viper is responsible for a substantial number of deaths in many South-East Asian countries including India. This venom is composed of a myriad of biologically active components predominated by pro-coagulant and anticoagulant proteases, phospholipase A_2, L-amino acid oxidase and many other toxins and proteins. The structure–function properties and mechanisms of action of several of such enzymes/toxins have been well characterized (Mukherjee et al., 2000; Doley and Mukherjee, 2003; Mukherjee, 2008; Saikia et al., 2011; Mukherjee and Mackessy, 2013). By mass spectroscopic analysis we have detected numerous low molecular mass (2–6 kDa) peptides in venom of Russell's viper; nevertheless, characterization of biological activity of majority of such molecules remains unexplored (A.K.

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Mukherjee, unpublished observation). Studies on such molecules may advance our knowledge on their contribution in RVV induced pathogenesis in addition to harnessing them as therapeutically important lead drug molecules. To the best of our knowledge, this is the first report on purification and characterization of a low molecular mass (3889.960 Da), pro-angiogenic novel peptide from RVV, named RVVAP.

2. Methods

2.1. Purification of a low molecular mass peptide from Russell's viper venom

Dry, pooled, desiccated *D. r. russelii* venom of eastern India origin was purchased from the licensed supplier (Mr. D. Mitra, Calcutta Snake Park, Kolkata). Crude RVV (20 mg) dissolved in 2.0 ml of 20 mM potassium(K)-phosphate buffer, pH 7.0 was applied to a CM-cellulose (20 × 60 mm) column pre-equilibrated with the above buffer. After washing the column with two volumes of equilibration buffer, the bound proteins were eluted stepwise using potassium-phosphate buffers of different molarities and pH values at room temperature (~23 °C). The flow rate was adjusted to 24 ml/h and 1.0 ml fraction was collected. Protein content of the individual fractions was determined by the method of Lowry et al. (1951).

The fractions eluted with 50 mM K-phosphate buffer, pH 7.0 were pooled (named CM-III fraction), desalted, concentrated and then loaded to a Sephadex G-50 gel filtration column (1 × 64 cm^2) equilibrated with 20 mM K-phosphate buffer, pH 7.0. The elution of proteins was carried out with the equilibration buffer at room temperature (~23 °C). Fractions showing angiogenic activity were pooled and further purified by reverse-phase (RP) HPLC (Waters) using a C_{18}-μ Nova-Pak column (150 mm × 300 mm) equilibrated with 0.1% (v/v) trifluroacetic acid (TFA, v/v) in 5.0% (v/v) acetonitrile. The proteins were eluted with a 5–95% (v/v) linear gradient of acetonitrile containing 0.1% TFA (v/v) over 10 min. Protein elution was monitored at 220 nm and fractions were collected manually, dried in vacuum and stored until further use.

The purity and molecular weight of major RP-HPLC peak protein was confirmed by matrix-assisted laser desorption/ionization time of flight-mass spectrometry (MALDI-TOF-MS). Partial N-terminal amino acid sequencing of purified protein by Edman degradation was performed using a gas phase PPSQ-10 protein sequencer (Shimadzu) connected to an on-line PTH analyzer and a CR-7A data processor. Protein homology searches were performed using the Swiss-Prot Data Bank (BLAST programme of NCBI). The secondary structure of purified protein was determined by circular dichroism (CD) spectrum (Jasco J715 Spectropolarimeter, Europe) as described previously (Doley et al., 2004). CDPRO CLUSTER software was used to determine the secondary structure of purified peptide.

2.2. Angiogenesis activity assay

2.2.1. Egg yolk angiogenesis assay

Fourth day pre-incubated eggs were collected from the Poultry Research Station, Nandanam, Chennai. Eggs were broken and gently plated on a cellophane bed in Petri dishes under sterile conditions. Discs containing 50 and 100 ng/ml of RVVAP were then placed on the *Area Vasculos* and incubated for 24 h. Images were taken at 0, 4, 8, 12 and 24 h of incubation under 20× magnification using a Nikon CoolPix camera adapted to a stereomicroscope. The quantification of angiogenesis was performed by using Angioquant software (Niemistö et al., 2005).

2.2.2. Wound healing assay

The angiogenesis inducing capacity of purified protein was determined by wound healing experiments. Briefly, one million EA.hy926 (human umbilical vein cell line) cells in 2.0 ml of Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) were seeded in a 12 wells plate and then incubated at 37 °C/5% CO_2. Twenty-four hours later, when the cells reached confluence, wound was created by scratching the monolayer with a 1 mm wide sterile plastic scraper. As per the experimental protocol described elsewhere (Staton et al., 2004), the cells were washed with 1× PBS, treated with purified RVVAP and incubated for another 8 h at 37 °C/5% CO_2. Bright-field images were taken with 4× magnification under an inverted bright field microscope. The rate of wound healing was quantified from the images using Scion Image, Release Alpha 4.0 3.2 and Adobe Photoshop version 6.0.

2.2.3. Endothelial ring formation assay

In 2D cultures, endothelial cells rearrange themselves to form endothelial rings, the cellular unit of angiogenesis (Sinha et al., 2011). EA.hy926 cells were seeded on collagen (collagen type I) plated 24-well plates. After 12 h of incubation at 37 °C/5% CO_2, cells were treated with RVVAP for 6 h under the above conditions. After incubation, the number of rings formed in the monolayer was counted under bright field phase contrast microscope and the phase contrast images were taken.

2.2.4. Cell migration assay

The migration assay was performed in a Boyden chemokinesis apparatus. EA.hy926 cells treated with various doses of RVVAP, were trypsinized with 0.25% trypsin and 2 × 10^4 cells/ml were suspended in 25 μl of DMEM with 10% (v/v) FBS and then 1% sterile solution of penicillin–streptomycin was added to the upper chamber of the Boyden apparatus. Polycarbonate membranes with 8 μm pores were coated with 0.4 μg/ml collagen (collagen type-I), washed twice with phosphate buffered saline, and placed above the wells of the lower chamber containing DMEM with 10% (v/v) FBS. The top half of the chamber was reattached, and the chamber was incubated at 37 °C/5% CO_2 for 2 h to allow a uniform cell attachment to the filter. After incubation, the filter was removed from the apparatus and the cells were fixed with 4% paraformaldehyde and counterstained with propidium iodide (1 μg/ml). The number of migrated cells on the lower surface of the membrane was counted in 6 randomly chosen fields under the Olympus IX71 inverted fluorescence microscope using a red filter (590 nm) at 4× magnification and averaged.