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A novel disintegrin protein from *Naja naja* venom induces cytotoxicity and apoptosis in human cancer cell lines *in vitro*

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ABSTRACT

Animal venoms and toxins are potential bioresources that have been known to mankind as a therapeutic tool for more than a century through folk and traditional medicine. The purified "disintegrin protein" (64 kDa) from the venom of the Indian cobra snake (*Naja naja*) exhibited cytotoxic effects of various types of human cancer cell lines such as breast cancer (MCF-7), lung cancer (A549) and liver cancer (HepG2). *In vitro* cytotoxicity, DNA fragmentation, an apoptotic assay and a cell cycle analysis were performed to evaluate the anticancer activity of disintegrin against the above cell lines. The IC_{50} value of disintegrin was determined to be 2.5 ± 0.5 μg/mL, 3.5 ± 0.5 μg/mL, and 3 ± 0.5 μg/mL for the MCF-7, A549 and HepG2 cell lines respectively. Moreover, the increased distribution of G0/G1 and S phase led to decreased populations of cells in the G2/M phase of MCF-7, HepG2 and A549 cells.

1. Introduction

Cancer is a complex disease that is characterized by cell proliferation (uncontrolled cell division), cell transformation and an escape from apoptosis and these properties pave the way for invasion, angiogenesis and metastasis. The use of snake venom as a drug has been known for many decades. It known that the physiologically active components of snake venom possess a therapeutic potential [1]. Snake venom is a complex mixture of proteins and peptides. Among these proteins and peptides, disintegrin forms a family of small, naturally occurring peptides containing 49–84 amino acid residues [2]. It has also been suggested that integrins play an important role in the intraperitoneal dissemination of ovarian cancer, as demonstrated by the finding that multiple intraperitoneal injections of a rat genome database (RGD) peptide inhibited the experimental peritoneal seeding of human ovarian cancer cells *in vivo* [3]. The results can be partially explained by the potent tumor apoptotic effects of integrin and their role in the arrest of the cell cycle.

Snake venom comprises complex mixtures of enzymatic and non-enzymatic proteins, peptides and small organic compounds, such as citrate, nucleosides and acetylcholine. Disintegrin plays an important role in many diverse biological processes, including platelet aggregation, inflammatory action and immune reactions. Disintegrin is an antagonist that inhibits the interaction of integrins with their individual ligands. Most of the disintegrins have a rat genome database (RGD) motif, which proposed to be involved in the recognition of the adhesive protein [4]. As previously described [5], the therapeutic value of snake venom (*Naja* sp.) has been widely exploited to target various types of cancer [6]. Cancerous cells produce reactive oxygen species (ROS) and other inflammatory mediators. Among these mediators, the ROS have been clearly demonstrated to knock out the tumor suppressor gene by following oncogene activation. It has been reported that the activity of the ROS scavenging system is altered in tumor cells [7]. The whole venoms of the Indian monocle cobra (*Naja kaouthia*) and Russell's viper (*Vipera russelli*) were reported to exhibit significant levels of anticancer activity against Ehrlich's Ascites Carcinoma (EAC) cells [8]. Snake venoms exhibit a spectrum of biological activities, which results in local and systemic toxicity. Work on the pharmacology and chemistry of venoms has shown that there is a considerable variability of venom composition even within a species [9]. Cytotoxin IIa, from the Indian cobra (*Naja naja*) venom exhibited

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cytotoxicity toward Yoshida sarcoma and ascites hepatoma cells. Three phospholipases from the venom of the cobra *N. naja* venom exhibited cytotoxic activity against Ehrlich's Ascites Carcinoma (EAC) cells [10].

In the present investigation, it is further confirmed that the *N. naja* venom protein, disintegrin, had cytotoxic activity against human breast cancer MCF-7 cells, liver cancer HepG2 cells and lung carcinoma A549 cells. The role disintegrin as a potent *in vitro* growth inhibitor of MCF-7, HepG2 and A549 cells and its underlying mechanism of inhibition of disintegrin were monitored through *via* the induction of apoptosis and the arrest of G0/G1 cell cycle arrest. This investigation is a significant step toward understanding the anticancer activity of the disintegrin protein isolated from the venom of the Indian cobra *N. naja*. Accordingly, the results of the present study introduce new avenues for designing and developing novel antineoplastic drugs from unconventional sources such as the snake venom.

2. Methods

2.1. Chemicals and reagents

Sodium bicarbonate, sodium pyruvate, L-glutamine, glucose, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES), penicillin and streptomycin were purchased from Sigma (USA). Dulbecco's minimal essential medium (DMEM) (Sigma, USA), eagles minimal essential medium (Hi-Media, India), calf insulin (Hi-Media, Inc), fetal bovine serum (GIBCO, USA) were also used in the present study.

2.2. Snake venom collection

The venom extraction from the Indian cobra (*N. naja)* was performed in the Department of Antitoxin, King Institute of Preventive Medicine and Research, Chennai, India, by allowing the snake to bite into parafilm stretched over a disposable plastic cup. The venom sample was centrifuged ($500 \times g$ for 10 min) and filtered through a 0.45 μm filter under positive pressure. The venom was frozen in a −80 °C deep freezer for 4 h and then lyophilized [8].

2.3. Cell culture

The cancer cell lines, MCF-7, A549 and HepG2 were purchased from the National Center for Cell Sciences (NCCS), Pune, India. The cancer cells were maintained in Dulbecco's modified eagles medium (DMEM) supplemented with 2 mM L-glutamine and balanced salt solution (BSS) adjusted to contain 1.5 g/L Na_2CO_3, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 1.5 g/L glucose, 10 mM (4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid) (HEPES) and 10% fetal bovine serum (GIBCO, USA). Penicillin and streptomycin (100 IU/100 μg) were adjusted to 1 mL/L. The cells were maintained at 37 °C with 5% CO_2 in a humidified CO_2 incubator [11,12].

2.4. Purification of disintegrin

The purification of disintegrin was performed in two successive steps that included ammonium sulfate precipitation and gel filtration chromatography. The freshly prepared venom was dissolved in the required volume of 10 mM Tris HCl buffer (pH 8.0) and then subjected to ammonium sulfate precipitation (20–60%). The resulting pellet 60% precipitation was dialyzed against the same buffer and then concentrated and lyophilized. Initially, the column was equilibrated with 50 mM Tris HCl (pH 8.0). The lyophilized cobra venom sample was loaded on top of the Sephadex G-100 column (90 cm) at 25 °C. Two milliliter fractions were collected. Among the various fractions, the most active and stable fractions were pooled, dialyzed and lyophilized.

2.5. SDS-PAGE for separation of disintegrin

SDS-PAGE (sodium do-decyl sulfate polyacrylamide gel electrophoresis) was performed using an electrophoresis unit (GENEI, India) with a 5% stacking gel and a 12% separating gel polyacrylamide in 25 mM Tris HCl, pH 8.3, 0.18 M glycine and 0.1% SDS. The separation was performed out at 35 mA for 5 h. After migration, the protein bands of the purified snake venom were stained with Coomassie brilliant blue for detection and the determination of purity.

2.6. Evaluation of cytotoxicity and IC_{50}

The inhibitory concentration (IC_{50}) value was evaluated using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Cancer cells were grown (1×10^4 cells/well) in a 96-well plate for 48 h in to 75% confluence. The medium was replaced with fresh medium containing serially diluted disintegrin, and the cells were further incubated for 48 h. The culture medium was removed, and 100 μL of the MTT [3-(4,5-dimethylthiozol-2-yl)-3,5-diphenyl tetrazolium bromide] (Hi-Media) solution was added to each well and incubated at 37 °C for 4 h. After removal of the supernatant, 50 μL of DMSO was added to each of the wells and incubated for 10 min to solubilize the formazan crystals. The optical density was measured at 620 nm in an ELISA multiwell plate reader (Thermo Multiskan EX, USA). The OD value was used to calculate the percentage of viability using the following formula [13]:

$$\%\ \text{of viability} = \frac{\text{OD value of experimental sample (disintegrin treated)}}{\text{OD value of experimental control (untreated cells)}} \times 100$$

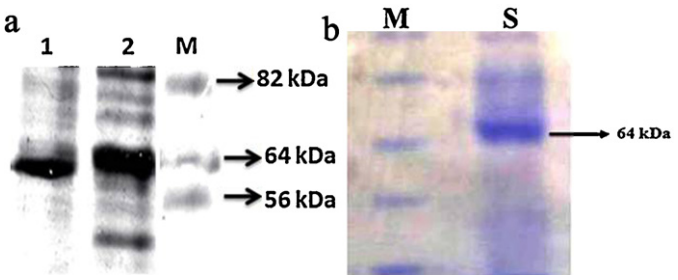

Fig. 1. (a) Purification of the disintegrin protein (lane 1) as shown in using SDS-PAGE. Samples containing 100 μg of *N. naja* whole venom (lane 2) and 50 μg of the Sephadex G-100 fraction. (b) Confirmation of disintegrin using the SDS-PAGE method indicates the confirmed 64 kDa protein in lane S and lane M represents the differential pattern of marker sizes.

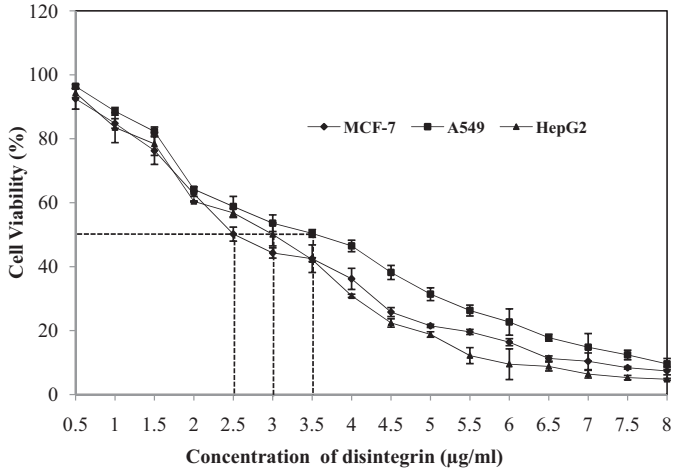

Fig. 2. The effect of different doses of disintegrin on growth induced cytotoxicity in selected cancer cells. After 48 h of induction by disintegrin, these cancer cells were exposed to different doses of disintegrin (0.5–8 μg) for 48 h. The detected IC_{50} concentrations were 2.5 ± 0.5 μg/mL, 3 ± 0.5 μg/mL and 3.5 ± 0.5 μg/mL for the MCF-7, HepG2 and A549 cells respectively. The data were expressed as the mean ± SD and $P < 0.05$ between the treated and control cells.

2.7. Morphological study

The MCF-7, A549 and HepG2 cells that were grown on cover slips (1×10^5 cells/cover slip) were incubated with disintegrin at the IC_{50} concentration, and they were then fixed in an ethanol:acetic acid solution (3:1, v/v). The cover slips were gently mounted on glass slides for the morphometric analysis. Three monolayers per experimental group were photo micrographed. The morphological changes of the MCF-7, A549 and HepG2 cells were analyzed using Nikon (Japan) bright field inverted light microscopy at 40× magnification.

2.8. Assessment of DNA fragmentation

MCF-7, A549 and HepG2 cells (10^6 cells) were independently suspended in 10 mL of buffer containing 10 mM Tris HCl and 10 mM EDTA (pH 8.0). The cells were then treated with 10 mL of a solution that contained 10 mM Tris HCl, 10 mM EDTA (pH 8.0), 2% SDS, and 20 mg/mL proteinase K. The mixture was incubated at 37 °C for 3 h, followed by DNA extraction with a phenol:chloroform:isoamyl alcohol solution (25:24:1). The DNA was treated with DNase free RNase at a concentration of 20 mg/mL at 4 °C for 45 min and precipitated with 100 mL of 2.5 M