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A cytotoxin isolated from Agkistrodon acutus snake venom induces apoptosis via Fas pathway in A549 cells

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Abstract

ACTX-6 is a protein isolated from Agkistrodon acutus snake venom and demonstrated cytotoxic activity to various cancer cells in vitro. In this paper the exact mechanism in ACTX-6-induced cell death was investigated and it was found that ACTX-6 could induce cell apoptosis. The results of Western blot and RT-PCR showed that ACTX-6 could induce Fas and FasL protein expression. When Fas signaling pathway was blocked by neutralizing antibodies to Fas or FasL, ACTX-6-induced apoptosis was inhibited. DISC formation was also detected by immunoprecipitation. These results suggested that Fas pathway was involved in ACTX-6-induced apoptosis. The activities of caspase-3, 8 and 9 were assayed and the activation of caspase-9 demonstrated that mitochondrial pathway was also involved in ACTX-6-induced apoptosis. Bid cleavage and dissipation of mitochondrial membrane potential ($\Delta\psi_m$) verified the involvement of mitochondria. ACTX-6 is an L-amino acid oxidase and can oxidize L-amino acid to generate hydrogen peroxide. The production of ROS in ACTX-6-treated cells was detected and the ROS scavenger catalase could inhibit ACTX-6-induced apoptosis. Western blot analysis showed that JNK was phosphorylated in ACTX-6-treated cells and c-Jun was also activated. JNK inhibitor SP600125 could inhibit ACTX-6-induced apoptosis and catalase could inhibit JNK and c-Jun phosphorylation. It could be concluded that JNK pathway was necessary in ACTX-6-induced apoptosis and the oxidative stress generated by ACTX-6 was responsible for the activation of JNK.
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Keywords: Apoptosis; Fas; Mitochondria; ROS: JNK

1. Introduction

Many reports have revealed that apoptosis induction is the most important mechanism of anticancer agents (Sun et al., 2004). Previous studies have shown that many anticancer agents, including chemotherapeutic agents, hormones, and various biological compounds, induced apoptosis in carcinoma cells in vitro (Kobayashi et al., 1994; Kaufmann and Earnshaw, 2000). Today many apoptosis-inducing factor has been found from animal toxins, such as apoxin I (Torii et al., 1997) and achacin (Kanzawa et al., 2004), both of which are extracted from snake venom. ACTX-6 is a 98 kDa protein containing two subunits isolated from Agkistrodon acutus snake venom in our laboratory (Zhang et al., 2004b). Previous studies have shown that ACTX-6 exhibits cytotoxic properties against many cancer cell lines in vitro, including A549, Hela, BGC, SMMC7721, KB and Caco-2 cells (Zhang et al., 2004b). In addition, it demonstrates L-amino acid oxidase (LAAO) activity in vitro. LAAO catalyzes the oxidative deamination of L-amino acids, producing the corresponding a-ketoacids, hydrogen peroxide, and ammonia. Many studies have reported that LAAO in snake venom is an apoptosis inducing factor, and the main mechanism of

Abbreviation: LAAO, L-amino acid oxidase; JNK, c-Jun NH2-terminal kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyltetrazodium bromide; DISC, death-inducing signaling complex; $\Delta\psi_m$,, mitochondrial transmembrane potential; DCF, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; ROS, reactive oxygen species; FADD, Fas-associated death domain; FasL, Fas ligand.

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cytotoxicity of LAAO is believed to depend on the generation of hydrogen peroxide (Torii et al., 2000; Suhr and Kim, 1999; Souza et al., 1999; Ali et al., 2000). In this paper, we try to investigate whether ACTX-6 is an apoptosis inducing factor and the role of hydrogen peroxide in ACTX-6-induced cell death.

2. Materials and methods

2.1. Cell cultures

The human lung cancer cell line A549 was maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2/95% air. ACTX-6 was dissolved in culture medium and in all experiments negative control cells were treated with medium without ACTX-6.

2.2. Assessment of cytotoxicity

The cytotoxic effect of ACTX-6 was evaluated by MTT method (Uddin et al., 1997). Briefly, 5×10^4 cells were seeded into a 96-well plate in triplicate and 6 h later a series of drugs were added into the wells at the indicated final concentrations. After incubation with drugs for 24 h, the medium in each well was replaced with 20 μL of MTT (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyltetrazodium bromide), 5 mg/mL final concentration, and 4 h later 100 μL DMSO/well were added to dissolve the formed violet formazan crystals within metabolically viable cells. The plates were incubated at 37 °C for 15 min and then read at 590 nm with a microplate reader. Percent cell viability was calculated as (experimental sample-blank)/(control-blank).

2.3. Phosphatidyl serine exposure

The annexin-V-fluorescein isothiocyanate (FITC) conjugated/propidium iodine (PI) assay (Molecular Probes, Eugene, OR, USA) was used to detect PS externalization. Fluorescence-activated cell sorter (FACS) analysis was performed on a FACScalibur flow cytometer (Becton–Dickinson). This assay discriminates viable cells (annexin-V^-/PI^-) from cells in early apoptosis (annexin-V^+/PI^-), cells in late apoptosis or secondary necrosis (annexin-V^+/PI^+), or cells undergoing necrosis (annexin-V^-/PI^+). A total of 10,000 cells were acquired per sample and the data were analyzed using Cell Quest software (Becton–Dickinson).

2.4. Western blot analysis

Cells (5×10^4/mL) were seeded in 6-well plates (2 mL/well) and after 6 h attachment, culture media were replaced with media containing indicated concentrations of drugs. The cells were treated for indicated time and non-treated cells served as controls. Then cells were lysed in lysis buffer (50 mM Hepes–KOH, pH 7.4, 250 mM NaCl, 1% NP-40, 5 mM DTT, protease inhibitors). Total protein concentrations were determined using Bio-Rad protein assay reagent according to the manufacturer's instructions. Equal amounts of proteins were separated by SDS–PAGE and transferred to a nitrocellulose membrane. The membranes were blocked for 1 h with 5% non-fat dry milk in PBS, 0.1% Tween 20 and incubated with the primary antibodies for Fas, FasL (Fas ligand), FADD, caspase-8, Bid, Actin (all from Boster, Wuhan, China), GAPDH (Protein Tech Group, Chicago, USA), phospho-JNK1 (St. Cruz, CA) and phospho-c-Jun (Cell Signaling, Beverly, MA) overnight at 4 °C. Then the membranes were incubated with appropriate secondary antibodies and were detected using ECL method (Amersham–Pharmacia Biotech, Uppsala, Sweden).

2.5. RNA isolation, RT-PCR and semi-quantitative PCR analysis

RT-PCR assays were used to assess Fas mRNA levels in A549 cells. Total RNA was isolated from cells treated by 10 μg/mL ACTX-6 for indicated time using Trizol (Invitrogen, Carlsbad, CA, USA). Total RNA was treated with DNase I (Ambion, Austin, TX, USA) for 30 min at 37 °C to remove any contaminated genomic DNA before RT-PCR. The quality and quantity of the RNA preparations were tested by agarose gel electrophoresis and measurement of absorption at 260 and 280 nm. The ratio of optical density at 260 and 280 nm was $\geqslant 1.8$ in all cases.

One microgram of total RNA was reverse-transcribed using the RT-PCR kit (Perkin–Elmer Applied Biosystems, Foster City, CA, USA). Aliquots were amplified in a DNA thermocycler (Stratagene, Heidelberg, Germany) with 2.5 U recombinant *Taq* polymerase (Sigma–Aldrich) as described previously (Eichhorst et al., 2000). Amplification products were separated by electrophoresis on 1.2% agarose gels. One microliter of cDNA was used per PCR with 15 μL *Taq* PCR Master Mix Kit (Qiagen) plus 1 μL oligonucleotide primer pair (20 μM). Primers used for human GAPDH (958 bp product size), Fas (582 bp product size), and FasL (376 bp product size) are:

hGAPDH-RT-FP: 5′-GAAGGTGAAGGTCGGAGTC-3′
hGAPDH-RT-RP: 5′-GCTGTAGCCAAATTCGTTGT-3′
hFas-RT-FP: 5′-AGGGAAGCGGTTTACG-3′
hFas-RT-RP: 5′-CTCCAGCAATAGTGGTGAT-3′
hFasL-RT-FP: 5′-TTGTTACAGGCACCGAGAA-3′
hFasL-RT-RP: 5′-CCATCCCTTAAATCCTCA-3′

Total volume was brought to 30 μL with distilled and deionized water. The reaction mixtures were incubated in a thermal cycler under the following conditions: 94 °C for 3 min for initial denaturation, then up to 30 cycles with 94 °C for 1 min for denaturation, 52 °C for 1 min for

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