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Antiproliferative activity and apoptosis-inducing mechanism of Concanavalin A on human melanoma A375 cells

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ABSTRACT

The objective of this study was to investigate the antiproliferative activity and apoptosis-inducing mechanism of Concanavalin A (ConA) on human melanoma A375 cells. We firstly simulated the three-dimensional structure of ConA. Subsequently, we found that ConA possessed remarkable antiproliferative effect on A375 cells. Further experimental data indicated that there was a link between its hemagglutinating activity, mannose-binding activity and antiproliferative activity. In addition, we showed that ConA induced A375 cell apoptosis in a caspase-dependent manner. Then, we demonstrated that the treatment of ConA caused mitochondrial transmembrane potential (MMP) collapse, cytochrome *c* release and caspase activation. In conclusion, we report for the first time that there may be a close correlation between carbohydrate-binding activity of ConA and its antiproliferative activity. Also, we demonstrate firstly that ConA induces A375 cell death in a caspase-dependent manner as well as through a mitochondrial apoptotic pathway.

Plant lectins are reversible carbohydrate-binding proteins or glycoproteins of non-immuno origin that can agglutinate cells or precipitate polysaccharides and glycoconjugates [1,2]. In the past few years, several hundred of these proteins have been purified and characterized in some detail with respect to their biochemical properties, carbohydrate-binding specificity and various biological activities [3]. Concanavalin A (ConA),[1] the first lectin with a mannose/glucose-binding specificity, was found in the jack bean (*Canavalia ensiformis*) [4]. Monomeric units of ConA that have been identified have a molecular weight of approximately 27,000 Da [5]. Between pH 2 and 5.5, ConA exists as a dimer of two covalently linked subunits, whereas at pH values above 5.5 a tetramer [6]. In addition, ConA is the first lectin for which the three-dimensional structure has been established and is being widely studied for a variety of biological activities such as antiproliferative activity in cancer cells [7].

Of note, the development of cancer can be associated with programmed cell death (PCD), which is an evolutionary conserved process that plays a crucial role in metazoan development [8]. Apoptosis, type I PCD, is characterized by condensation of the cytoplasm and nucleus, DNA fragmentation, chromatin merging in the nuclear periphery, cell contraction, dynamic membrane blebbing, as well as cell phagocytosis. Additionally, apoptosis is also known as one of the most important mechanisms that can be regulated by numerous cellular signaling pathways for tumor cell suicide [9].

Previous studies have reported that ConA was cytotoxic or inhibitory to several typical tumor cell lines [10]; however, the precise mechanism by which it induces tumor cell death is still only rudimentarily understood. In the present study, we reported the mannose-binding activity of ConA had a significant influence on the antiproliferative activity, indicating that there is a close correlation between them. Subsequently, we indicated that ConA induced human melanoma A375 apoptosis in a caspase-dependent manner. Ultimately, we also demonstrated that ConA induced human melanoma A375 cell death through a mitochondrial apoptotic pathway.

Materials and methods

Molecular modeling

The structural data of ConA were acquired from the GenBank database (PDB code: 3cna). MODELLERv7 [11] was utilized to build the molecular modeling of ConA.

Reagents

ConA was purchased from Sigma Chemical (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from TBD Biotechnology Development (Tianjin, China); 3-(4,5-dimethylthiazol-2-yl)-2,5-

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[1] *Abbreviations used:* ConA, concanavalin A; MMP, mitochondrial transmembrane potential; PCD, programmed cell death; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly ADP-ribose polymerase; ICAD, inhibitor of caspase-activated DNase; LDH, lactate dehydrogenase.inhibitor of caspase-activated DNase.

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diphenyltetrazolium bromide (MTT), NBSF, 2,3-butanedione, PMSF, TNBS, DEPC, Man-α(1,3)-Man, Man-α(1,6)-Man and Man-α(1,3:1,6)-mannotriose were purchased from Sigma Chemical (St. Louis, MO, USA). Pancaspase inhibitor z-VAD-fmk, caspase-3 inhibitor z-DEVD-fmk, caspase-8 inhibitor z-IETD-fmk and caspase-9 inhibitor z-LEHD-fmk were purchased from Enzyme Systems (CA, USA). Rabbit polyclonal antibodies against caspase-3, caspase-9, poly ADP-ribose polymerase (PARP), inhibitor of caspase-activated DNase (ICAD) and β-actin, mouse polyclonal antibodies against cytochrome *c* and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

The human melanoma A375 cell lines were provided by Medical Sciences Center of West China of Sichuan University. The cells were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS) and 0.03% L-glutamine (GIBCO) and maintained at 37 °C with 5% CO_2 in a humidified atmosphere.

Cell growth inhibition assay

The human melanoma A375 cells were dispensed in 96-well flat-bottomed microtiter plates (NUNC, Roskilde, Denmark) at a density of 5×10^4 cells/ml. After 24 h incubation, the cells were treated with or without various inhibitors at given concentrations 1 h prior to the administration of the ConA. The effects of ConA on cell viability/proliferation were determined using the MTT assay with a plate reader (Bio-Rad, Hercules, CA, USA) as described previously [12].

Chemical modification assay

Hemagglutinating assays were performed in 96-well microtiter U plates according to serial dilution method with some modifications [13]. Modifications of Ser/Thr and His residues were used with PMSF and DEPC, respectively. Modification of Tyr residues was used with NBSFM. Modification of ε-NH_2 residues was used by 2,3-butanedione. Modification of Trp residues was used with TNBS.

Carbohydrate-binding activity assay

To assess the effect of carbohydrates on ConA-induced human melanoma A375 cell death, the MTT assay was determined as above except that the lectin was pre-incubated at 37 °C for 30 min with mannose (1-3), mannose (1-6) and mannose (1-3; 1-6) that completely inhibited the hemagglutinating activity of ConA at 24 h.

Microscopic observations of apoptotic changes

The human melanoma A375 cells were incubated with 25 μg/ml ConA for 24 h. The cellular ultrastructure was observed under transmission electron microscope (Hitachi 7000, Japan).

Lactate dehydrogenase (LDH) activity-based cytotoxicity assay

LDH activity was assessed by using a standardized kinetic determination kit (Zhongsheng, LDH kit, Beijing, China) as the method previously described with some modifications [14].

Measurement of Sub-G1 cells

Cell cycle and Sub-G1 distribution were determined by staining DNA with PI as previously described with some modifications [15].

Caspase assays

Caspase-3, -8 and -9 activities were measured by a colorimetric assay kit (Biovision) according to the manufacturer's instructions. Briefly, cell lysate from 1×10^6 cells was incubated at 37 °C for 2 h with 200 μM DEVD-pNA (caspase-3 substrate), IETD-pNA (caspase-8 substrate) or LEHD-pNA (caspase-9 substrate). Samples were read at 405 nm in a microplate reader (Bio-Tek Instruments) and expressed as fold increase on the basal level (DMSO-treated cells).

Detection of mitochondrial membrane potential

After incubation with ConA for the indicated time periods, the cells were stained with 1 μg/ml rhodamine 123 and incubated for 15 min at 37 °C. The fluorescence intensity of cells was measured by flow cytometry (Becton Dickinson, Franklin Lakes, NJ) [16].

Western blot analysis

The human melanoma A375 cells were treated with 25 μg/ml ConA for 0, 6, 12 and 24 h. Both adherent and floating cells were collected, and then Western blot analysis was carried out by the method as described previously [17].

Statistical analysis

All the data were confirmed in at least three independent experiments. These data were expressed as means ± SD. Statistical comparisons were made by Student's *t-test*. $P < 0.05$ was considered statistically significant.

Results

Molecular modeling of ConA

Due to the available structure data, we firstly built the three-dimensional structure of ConA monomer. As shown in Fig. 1, ConA exhibits the canonical 12-stranded β-sandwich structure.

Antiproliferative effect of ConA on A375 cells

The antiproliferative effect of ConA on human melanoma A375 cells was evaluated by the MTT assay. As shown in Fig. 2A, the exposure to ConA caused inhibition of human melanoma A375 cell growth in a time- and dose-dependent manner. ConA from 5 to 80 μg/ml exerted cytotoxic effect on A375 cell growth and treatment with 25 μg/ml ConA for 24 h resulted in almost 50% inhibition, suggesting that the IC_{50} value of ConA was 25 μg/ml for 24 h.

Effects of chemical modification on antiproliferative activity

Modifications of Ser/Thr and His residues did not produce any significant alteration in the hemagglutinating activity of ConA. Chemical modification of Tyr residues of ConA with NBSF produced significant alteration in the hemagglutinating activity and it resulted in more than 90% decrease in its hemagglutinating activity. The modification of ε-NH_2 residues by 2,3-butanedione resulted in 50% decrease in the hemagglutinating activity. Modification of Trp residues using TNBS resulted in approximately 95% decrease in the agglutination activity (shown in Fig. 2B-a). After the treatments of ConA were modified with these above-mentioned chemical agents, they were subsequently added into the A375 cells, respectively. Interestingly, the antiproliferative activity of ConA was shown to