Norcantharidin (NCTD) is the demethylated form of cantharidin, which is the active substance of mylabris. To examine the pathway of NCTD-induced A375-S2 cell death, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, photomicroscopical observation, DNA agarose gel electrophoresis, caspase activity assay and Western blot analysis were carried out. A375-S2 cells treated with NCTD exhibited several typical characteristics of apoptosis. The inhibitory effect of NCTD on human melanoma, A375-S2 cells, was partially reversed by the inhibitors of pan-caspase, caspase-3 and caspase-9. The activities of caspase-3 and -9 were significantly increased after treatment with NCTD at different time. The expression of inhibitor of caspase-activated DNase was decreased in a time-dependent manner, simultaneously, the ratio of Bcl-2/Bax or Bcl-xL/Bax was decreased and the expression ratio of proteins could be reversed by caspase-3 inhibitor. The expression of cytochrome c in cytosol was increased after NCTD treatment and caspase-3 inhibitor had no significant effect on the up-regulation of cytochrome c. These results suggest that NCTD induced A375-S2 cell apoptosis and the activation of caspase and mitochondrial pathway were involved in the process of NCTD-induced A375-S2 cell apoptosis.

Key Words: Cantharidin; Norcantharidin; Cell Line, Tumor; A375-S2 Cells; Apoptosis; Caspase; Mitochondria; Proto-Oncogene Proteins c-bcl-2; Cytochromes c

Received: 16 January 2004
Accepted: 20 April 2004

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**MATERIALS AND METHODS**

**Chemical reagents**

NCTD of analytical grade purity was from the Junan Pharmaceutical Works (Junan, China) and dissolved in RPMI-1640 (HyClone, U.S.A.). Caspase-8 inhibitor (z-IETD-fmk) was from Enzyme Systems (CA, U.S.A.). Caspase-3 inhibitor (z-DEVD-fmk) and pan-caspase inhibitor (z-VAD-fmk) were from Calbiochem (CA, U.S.A.). Caspase-9 inhibitor (Ac-LEHD-CHO), rabbit polyclonal antibodies against ICAD, cytochrome c, Bax and Bcl-xL, mouse polyclonal antibodies against Bcl-2, horseradish peroxidase-conjugated secondary antibody (goat-anti-rabbit or goat-anti-mouse) were from Santa Cruz Biotechnology (Santa Cruz, CA). Caspase-3, -8 and -9 Apoptosis Detection Kits were from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell culture**

A375-S2, melanoma cells, were obtained from American Type Culture Collection (ATCC, #CRL, 1872, MD, U.S.A.) and were cultured in RPMI-1640 medium (HyClone, U.S.A.) supplemented with 10% heat inactivated (56 °C, 30 min) fetal calf serum (Beijing Yuanheng Shengma Research Institution of Biotechnology, Beijing, China), 2 M L-glutamin (GIBCO, U.S.A.), 100 kU/L penicillin and 100 g/L streptomycin (GIBCO, U.S.A.) at 37 °C in 5% CO₂.

**Cell growth inhibition test**

A375-S2 cells (1.0 × 10⁶ cells/L) seeded in 96-well plate (NUNK™, Roskilde, Denmark) were cultured for 24 hr, then various concentrations of NCTD (60-480 µM) were added and cultured for 12, 24, 36, 48 hr further. MTT (thiazolyl blue, Sigma, MO, U.S.A.) test were carried out to detect cell growth using an enzyme-linked immunosorbent assay plate reader (TECAN, Austria) (18). After preincubation with given concentrations of pan-caspase inhibitor (z-VAD-fmk), caspase-8 inhibitor (z-IETD-fmk), caspase-9 inhibitor (Ac-LEHD-CHO), caspase-3 inhibitor (z-DEVd-fmk) for 2 hr, 60 µM NCTD were added and cultured for further 24 hr. Growth inhibition was evaluated by MTT method. The percentage of cell growth inhibition was calculated as follows:

\[
\text{Relative viability} \left(\%\right) = \frac{1 - \left[A_{492\text{ (control)}} - A_{492\text{ (NCTD)}}\right]}{A_{492\text{ (control)}}} \times 100
\]

**Nuclear damage observed by Hoechst 33258 staining**

Apoptotic nuclear morphology was assessed using Hoechst 33258 staining (Sigma, U.S.A.) as previously described (19). A375-S2 cells, containing adherent and floating, were collected by centrifugation at 1,000 g for 5 min, washed two times with PBS. The cells were fixed with 3.7% parafomaldehyde at room temperature for 2 hr, then washed and stained with Hoechst 33258 167 µM at 37 °C for 30 min. At the end of incubation, the cells were washed and resuspended in PBS for observation of nuclear morphology using fluorescence microscope (Nikon, Osaka, Japan).

**Lactate dehydrogenase (LDH) activity-based cytotoxicity assays (20, 21)**

The cells were cultured with NCTD for 12, 24 or 36 hr. Floating dead cells were collected from culture medium by centrifugation (240 g for 10 min at 4 °C), and the lactate dehydrogenase (LDH) content from the pellets lysed in 1% NP-40 for 15 min was used as an index of apoptotic cell death (LDHp). The released LDH in the culture medium (extracellular LDH or LDHe) was used as an index of necrotic cell death. The adherent and viable cells were lysed in 1% NP40 for 15 min to release LDH (intracellular LDH or LDHi). Then the substrate reaction buffer of LDH (L (+)-lactic acid 0.5 mM, indonitrotetrazolium 0.66 mM, phenazine methosulfate 0.28 mM, β-nicotinamide adenine dinucleotide 1.3 mM in pH 8.2 Tris-HCl) was added. The OD value at 492 nm of reaction for 1 and 5 min were assayed and LDH activities were determined by the average difference between 1 min and 5 min. The percentage of apoptotic and necrotic cell death was calculated as follows:

\[
\text{% apoptosis} = \frac{\text{LDHp}(\text{LDHp+LDHe+LDHi})}{100}
\]

\[
\text{% necrosis} = \frac{\text{LDHe}(\text{LDHp+LDHe+LDHi})}{100}
\]

**Determination of DNA fragmentation by agarose gel electrophoresis**

DNA extraction and electrophoresis were performed as described previously (22). In brief, A375-S2 cells, containing adherent and floating, were collected by centrifugation at 1,000 g for 5 min. The cell pellet was suspended in cell lysis buffer [Tris-HCl 10 mM (pH 7.4), EDTA 10 mM (pH 8.0), Triton-100 0.5%] and kept at 4°C for 10 min. The lysate was centrifuged at 25,000 g for 20 min. The supernatant was incubated with RNase A 40 µg/L (Sigma) at 37°C for 1 hr, then incubated with proteinase K 40 µg/L (Merck) at 37°C for 1 hr.
1 hr. The supernatant was mixed with NaCl 0.5 M and 50% 2-propanol at -20°C overnight, then centrifuged at 25,000 g for 15 min. After drying, DNA was dissolved in TE buffer [Tris-HCl 10 mM (pH 7.4), EDTA 1 mM (pH 8.0)] and separated by 2% agarose gel electrophoresis at 100 V for 1 hr.

Assay of caspase activities

A375-S2 cells were treated with or without NCTD. Analysis of caspase-3, caspase-8 and caspase-9 activities was performed using Caspase Apoptosis Detection Kit (Santa Cruz, CA, U.S.A.) according to the manufacturer’s instruction. In brief, harvested cells at various time points were washed with PBS two times and centrifuged at 150 g for 5 min. The supernatant was aspirated off and 100 μL cell lysis buffer (provided) was added to an Eppendorf centrifuge at 500 μL per 1 × 10⁶ cells. Cells in the lysis buffer were incubated on ice for 10 min. Reaction buffer containing 10 μL DTT, 10 μL of DEVD-AFC, IEVD-AFC or LEHD-AFC substrates and 380 μL H₂O was added to each aliquot of cell lysate. The reaction mixtures were incubated at 37°C for 1 hr. The fluorescence of the cleaved substrates was determined with a spectrofluorometer set at 400 nm excitation wavelength and at 505 nm emission wavelength. The unit of enzyme activity corresponds to the activity that cleaves the respective substrate in 1 min/ mg protein at 37°C.

Western blot analysis

A375-S2 cells were treated with 60 μM NCTD for 0, 12, 24, 36 hr. Both adherent and floating cells were collected and frozen at -80°C. Western blot analysis was performed as previously described (23) with some modification. Briefly, A375-S2 cells were lysed for 1 hr on ice in lysis buffer [50 mM HEPES (pH 7.4), 1% Triton X-100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), supplemented with proteinase inhibitors: 100 μg/mL aprotinin, 10 μg/mL leupeptin, and 100 μg/mL pepstatin]. Protein concentration was determined by the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA). The lysate was centrifuged at 16,000 g at 4°C for 10 min. Equivalent amounts of protein lysates were mixed in 2 × loading buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 10% 2-mercaptoethanol, 10% glycerol, and 0.002% bromphenol blue], heated at 100°C for 5 min, and then analyzed by electrophoresis in 12% SDS polyacrylamide gel and blotted onto nitrocellulose membrane (Amersham Biosciences, U.K.). After blocked with Tween 20-Tris-buffer saline [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.02% Tween 20] containing 5% nonfat milk at room temperature with the primary antibodies at 1:500 dilution in blotting buffer. After washed 3 times for 10 min each in Tris-buffered saline, the membrane was incubated with a diluted horseradish peroxidase-labeled secondary antibody (1:500) in blotting buffer at room temperature for 1 hr. After 3 more washes, proteins were detected by chemiluminescence, according to the manufacturer’s instructions (Bio-Rad Laboratories, Hercules, CA).

Statistical Analysis

All results were confirmed in at least three separate experiments. Data are expressed as mean ± SD. Data of the representative were analyzed for statistical significance by Student’s t test. p-value of less than 0.05 was considered statistically significant.
RESULTS

Cytotoxicity of NCTD on A375-S2 cells by MTT assay

NCTD 15 to 240 μM exerted potent inhibitory effect on A375-S2 cell growth. By 24 hr after NCTD 60 μM treatment, cell death rate reached to almost 50% (Fig. 2).

NCTD-induced morphological changes and DNA fragmentation of A375-S2 cells

In control group, A375-S2 cells were round in shape and stained homogeneously. After 24 hr treatment with NCTD, blebbing nuclei and granular apoptotic bodies appeared (Fig. 3, arrows).

DNA fragmentation as a hallmark of apoptosis was observed...
Effect of caspases on NCTD-induced cytotoxicity in A375-S2 cells

A375-S2 cells were treated with 60 μM NCTD for 24 hr in the absence or presence of various caspase inhibitors: pan-caspase inhibitor (z-VAD-fmk, 40 μM), caspase-3 inhibitor (z-DEVD-fmk, 20 μM), caspase-8 inhibitor (z-IETD-fmk, 20 μM), caspase-9 inhibitor (Ac-LEHD-CHO, 20 μM), Z-VAD-fmk, z-DEVD-fmk and Ac-LEHD-CHO partially blocked NCTD-induced A375-S2 cell apoptosis. Inhibitory ratio was 30.98%, 38.96%, and 51.50%, respectively. However, caspase-8 inhibitor did not affect the death ratio (Fig. 6). Caspase-3 activity was significantly enhanced after the cells were treated with NCTD 60 μM at different time points, however, caspase-9 activity increased moderately and increase of caspase-8 activity was almost negligible (Fig. 7).

The ICAD protein degradation was significant after 24 hr incubation with NCTD (Fig. 8), and this change was blocked by caspase-3 inhibitor.

Involvement of mitochondrial proteins in NCTD-induced A375-S2 cell apoptosis

After incubation with NCTD, protein expression ratio of Bcl-xL/Bax and Bcl-2/Bax was down-regulated, and this change was blocked by caspase-3 inhibitor. At the same time, the protein of cytochrome c was increased in cytosol and caspase-3 inhibitor had no influence on the increase (Fig. 9).

DISCUSSION

The present study showed that NCTD inhibited A375-S2 cell growth in a time- and dose-dependent manner. At the same time we demonstrated that NCTD induced apoptosis in A375-S2 cells and the apoptosis was possibly associated with caspases. Caspases are a family of cysteine proteases that are activated during the apoptotic processes. Death receptors such as Fas induce caspase-8 activation via Fas-associated death domain protein (FADD). It was reported that in human colorectal carcinoma cell lines NCTD induced apoptosis by activation of caspase-8, which was prevented by
the pan-caspase inhibitor z-VAD-fmk and caspase-8 inhibitor z-IETD-fmk (1), but the inhibition pathways of NCTD-induced apoptosis remain unclear. Morphological observation, DNA fragmentation and LDH activity assay suggested that NCTD induced A375-S2 cell death involved a mechanism of apoptosis. In the present study, NCTD-induced A375-S2 cell death was blocked by pan-caspase inhibitor, indicating that caspase family play a role in the apoptotic process. Caspase-3, and caspase-9 inhibitor (z-DEVD-fmk, Ac-LEHD-CHO, respectively) partially blocked NCTD-induced A375-S2 cell apoptosis, whereas caspase-8 inhibitor (z-IETD-fmk) had no effect on A375-S2 cell death induced by NCTD, indicating that post-mitochondrial caspase-9, but not upper stream caspase-8, activated this apoptotic process.

Chemotherapeutic agents and UV irradiation cause release of mitochondrial cytochrome c, which binds to Apaf-1, and this complex mediates recruitment of procaspase-9 and activates caspase-3. ICAD is expressed as two isoforms, ICAD-L/DD45 and ICAD-S/DDF35. Once ICAD/DDF45 is cleaved by caspase-3 or caspase-7, CAD is released to the nucleus and induces DNA fragmentation, resulting in the morphological and biochemical features of apoptosis (9, 10, 20, 24, 25). To further confirm the participation of different caspases in the cell death, we examined the activities of caspase-3, -8, and -9 and the protein expression of cytochrome c in the cell death, we examined the activities of caspase-3, -8, and -9 and the protein expression of cytochrome c in the cell death, we examined the activities of caspase-3, -8, and -9 and the protein expression of cytochrome c in the cell death, we examined the activities of caspase-3, -8, and -9 and the protein expression of cytochrome c in the cell death, we examined the activities of caspase-3, -8, and -9 and the protein expression of cytochrome c in the cell death, we examined the activities of caspase-3, -8, and -9 and the protein expression of cytochrome c in the cell death. These results suggested that the mitochondrial pathway of cell death, including Bcl-2 family and cytochrome c, might be involved in A375-S2 cell death and orchestrate the caspase cascades.

In conclusion, NCTD inhibited A375-S2 cell growth and caspase-9, caspase-3 activation was involved in the apoptotic progression. Simultaneously mitochondrial pathway, including cytochrome c, Bax and Bcl-2 (or Bcl-xL), contributed to the NCTD-induced A375-S2 cell apoptosis. More detailed mechanism of NCTD-induced A375-S2 cell apoptosis remains to be elucidated.

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