Norcantharidin induces apoptosis in HeLa cells through caspase, MAPK, and mitochondrial pathways

Wei-wei AN, Xian-feng GONG, Min-wei WANG[1], Shin-ichi TASHIRO[2], Satoshi ONODERA[2], Takashi IKEJIMA[3]

China-Japan Research Institute of Medical and Pharmaceutical Sciences,
Shenyang Pharmaceutical University,Shenyang 110016,China;
[1]Department of Pharmacology, Shenyang Pharmaceutical University, Shenyang 110016, China;
[2]Department of Clinical and Biomedical Sciences, Showa Pharmaceutical University, Tokyo 19-8543, Japan

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ABSTRACT

AIM: To investigate the mechanism of norcantharidin (NCTD)-induced HeLa cell apoptosis. **METHODS:** HeLa cell growth inhibition was measured by MTT method. Apoptosis was detected by Hoechst 33258 staining and agarose gel electrophoresis. Caspase activities were assayed using caspase apoptosis detection kit. Western blot analysis was used to evaluate the level of ICAD, ERK/p-ERK, JNK/p-JNK, and Bcl-X_L/Bax expression. **RESULTS:** Norcantharidin inhibited HeLa cell growth in a time- and dose-dependent manner. HeLa cells treated with norcantharidin showed typical characteristics of apoptosis including the morphological changes and DNA fragmentation. Caspase family inhibitor (z-VAD-fmk), caspase-8, -9 inhibitor (z-IETD-fmk, Ac-LEHD-CHO, respectively) and caspase-3 inhibitor (z-DEVD-fmk) partially prevent norcantharidin-induced apoptosis, but initiator caspase-1 inhibitor (Ac-YVAD-fmk) did not. The activities of caspase-3, -8, and -9 were up-regulated after norcantharidin treatment. Furthermore, NCTD–induced activation of caspase-3 resulted in the degradation of the inhibitor of caspase-activated DNase (ICAD). Up-regulation of mitochondrial Bax expression and down-regulation of Bcl-x_L expression also participated in the apoptosis induced by NCTD. Although p38 MAPK inhibitor (SB203580) failed to block cell death, ERK MAPK inhibitor (PD98059) and JNK MAPK inhibitor (SP600125) had marked inhibitory effects on norcantharidin-induced apoptosis. Moreover, the phosphorylation of JNK were up-regulated followed by delayed ERK phosphorylation after treatment with NCTD, suggesting that ERK and JNK were both responsible for NCTD-induced apoptosis in HeLa cells and worked at different stages. **CONCLUSION:** The cytotoxic effect of NCTD on HeLa cells was mainly due to apoptosis. The anti-tumor mechanism of NCTD might involve caspses, mitochondrial, and MAPKs pathways.

INTRODUCTION

Mylabris, the dried body of the Chinese blister beetle (*Mylabris Phalerata* Pallas), has been used as Chinese medicine for over 2000 years. Its active constituent, cantharidin, has antitumor properties and causes leukocytosis. Norcantharidin (NCTD), the demethylated form of cantharidin, is easier to be synthesized and is relatively free from side effects. The induction of apoptosis by NCTD on a variety of cancer cells such as HL-60, K562, Bel-7402, MCF-7 has been reported[1]. However, the exact mechanism responsible

[3] Correspondence to Prof Takashi IKEJIMA.
Phn & Fax 86-24-2384-4463. E-mail ikejimat@vip.sina.com
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for the apoptotic effect is not thoroughly elucidated.

Apoptosis, also known as programmed cell death, plays a critical role in embryogenesis, carcinogenesis and virally infected cell death. In the apoptotic process many proteins and genes are involved[2-4]. Cysteine-dependent aspartate-specific proteases (caspases), mitochondrial proteins of the Bcl-2 family and mitogen-activated protein kinase (MAPK) family, including ERK, JNK and p38, are important mediators of signal transduction in cell apoptosis. Caspases mediate apoptosis by proteolysis of specific substrates including inhibitor of caspase-activated DNase (ICAD), poly (ADP-ribose) polymerase-1 (PARP) and anti-apoptotic mitochondrial protein, Bcl-2[5-8]. It has been reported that ERK plays essential role in the control of cell growth and differentiation. In contrast, the activation of JNK and p38 MAPK is associated with induction of apoptosis[9-11].

In the present study, we demonstrated that caspases and Bcl-2 family proteins participated in the apoptosis induced by NCTD. In addition, the activation of ERK and JNK MAPK at different time stages contributed to NCTD-induced cell death.

MATERIALS AND METHODS

Chemical reagents NCTD of analytical grade purity was purchased from the Ju-nan Pharmaceutical Works (Junan, China) and was dissolved in RPMI-1640 (HyClone, USA). Caspase family inhibitor (z-VAD-fmk), and caspase-8 inhibitor, (z-IETD-fmk) were purchased from Enzyme Systems (CA, USA). Caspase-1 inhibitor (Ac-YVAD-cmk) was obtained from Bachem (Bubendorf Swizerland). Caspase-3 inhibitor (z-DEVD-fmk), ERK MAPK inhibitor (PD98059), p38 MAPK inhibitor (SB203580) and JNK MAPK inhibitor (SP600125) were purchased from Calbiochem (CA, US). Caspase-9 inhibitor (Ac-LEHD-CHO), rabbit polyclonal antibodies against ICAD, p38, ERK, JNK, phosphor-ERK, phosphor-JNK, and horseradish peroxidase-conjugated secondary antibody (goat-anti-rabbit) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-Bcl-x_L and anti-Bax were from Oncogene Research Products (MA, USA). Rabbit polyclonal anti-phosphor-p38 was from Techne. Caspase-3, -8, and -9 Apoptosis Detection Kit was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture HeLa, human cervical cells, were obtained from American Type Culture Collection (ATCC, #CRL, 1872, MD, USA) and were cultured in RPMI-1640 medium (HyClone, USA) supplemented with 10 % heat inactivated fetal calf serum (Beijing Yuanheng Shengma Research Institution of Biotechnology, Beijing, China), L-glutamin 2 mmol/L (GIBCO), penicillin 100 kU/L and streptomycin 100 g/L (GIBCO, USA) at 37 ºC in 5 % CO_2.

Cell growth inhibition test HeLa cells seeded in 96-well plate (NUNK^{TM}, Denmark) were cultured for 24 h, then various concentrations of NCTD (60-480 μmol/L) were added and cultured for 12, 24, and 48 h. MTT (Thiazolyl blue, Sigma, MO, USA) test were performed to detect cell growth using an enzyme-linked immunosorbent assay plate reader (TECAN, Austria)[12]. After preincubation with given concentrations of MAPK inhibitors, caspase family inhibitor (z-VAD-fmk), caspase-1 inhibitor (Ac-YVAD-cmk), caspase-8 inhibitor, (z-IETD-fmk), caspase-9 inhibitor (Ac-LEHD-CHO), caspase-3 inhibitor (z-DEVD-fmk), ERK MAPK inhibitor (PD98059), p38 MAPK inhibitor (SB203580) or JNK MAPK inhibitor (SP600125) for 2 h, NCTD 120 μmol/L were added and cultured for further 24 h. Growth inhibition was evaluated by MTT method. The percentage of cell growth inhibition was calculated as follows:

Inhibition (%)=[A_{492}(control)–A_{492}(drug)]/A_{492} (control)×100

Nuclear damage observed by Hoechst 33258 staining Apoptotic nuclear morphology was assessed using Hoechst 33258 (Sigma, USA) as described previously[13]. HeLa cells, containing adherent and floating, were collected by centrifugation at 1000×*g* for 5 min, washed two times with PBS. The cells were fixed with 3.7 % paraformaldehyde for 2 h at room temperature, then washed and stained with Hoechst 33258 167 μmol/L for 30 min at 37 ºC. At the end of incubation, the cells were washed and resuspended in PBS for observation of nuclear morphology using fluorescence microscope (Nikon, Japan).

Determination of DNA fragmentation by agarose gel electrophoresis DNA extraction and electrophoresis were performed as described previously[14]. In brief, HeLa cells, containing adherent and floating, were collected by centrifugation at 1000×*g* for 5 min. The cell pellet was suspended in cell lysis buffer (Tris-HCl 10 mmol/L pH 7.4, edetic acid 10 mmol/L pH 8.0, Triton-100 0.5 %) and kept at 4 ºC 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, USA)

at 37 ºC for 60 min, then incubated with proteinase K 40 μg/L (Merck, USA) at 37 ºC for 60 min. The supernatant was mixed with NaCl 0.5 mol/L and 50 % 2-propanol at -20 ºC overnight, then centrifuged at 25 000×*g* for 15 min. After drying, DNA was dissolved in TE buffer (Tris-HCl 10 mmol/L pH 7.4, edetic acid 1 mmol/L pH 8.0) and separated by 2 % agarose gel electrophoresis at 100 V for 60 min.

Caspase-3, -8, and -9 activity assay HeLa cells were treated with NCTD 120 μmol/L for 0, 8, 18, and 28 h. Analysis of caspase-3, caspase-8, and caspase-9 activities was performed using Caspase Apoptosis Detection Kit (Santa Cruz, CA) according to the manufacturer's instruction. In brief, cells (1×10^6) were pelleted by centrifugation, washed with PBS two times and incubated in 500 μL lysis buffer on ice for 10 min, then 1×reaction buffer and 10 μL DEVD-AFC, IEVD-AFC or LEHD-AFC substrates was added to lysis buffer. The reaction mixtures were incubated at 37 ºC for 60 min. Activities of caspase-3, -8, and -9 were measured by spectrofluorometry.

Western blot analysis HeLa cells were treated with 120 μmol/L NCTD for 0, 12, 24, 36, and 48 h. Both adherent and floating cells were collected and frozen at -80 ºC. Western blot analysis was performed as previously described[15] with some modification. The protein concentration was determined by the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA). Equivalent amounts of protein lysates were separated by electrophoresis in 12 % SDS polyacrylamide gel and blotted onto nitrocellulose membrane (Amersham Biosciences, UK). Protein expression was detected using primary polyclonal antibody against ICAD, Bcl-x_L, Bax, ERK, phosphor-ERK, JNK, phosphor-JNK, p38, phosphor-p38 and secondary polyclonal antibody conjugated with peroxidase.

Statistical analysis All results were confirmed in at least three separate experiments. Data were expressed as mean±SD. Data of the representative were analyzed for statistical significance by *t*-test. $P<0.05$ was considered statistically significant.

RESULTS

Cell growth inhibition of NCTD on HeLa cells NCTD induced cell death in dose- and time-dependent manners in HeLa cells. NCTD 60 to 480 μmol/L exerted potent inhibitory effect on HeLa cell growth. By 24 h after NCTD 120 μmol/L treatment, cell growth inhibition rate reached almost 60 % (Fig 1).

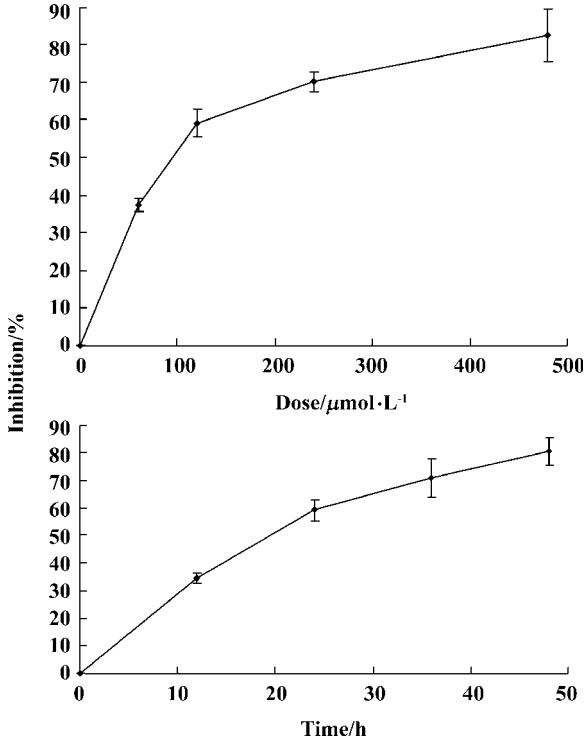

Fig 1. Dose- and time-dependent inhibition of proliferation of HeLa cells by NCTD treatment. (A) HeLa cells were treated with various doses of NCTD for 24 h. (B) The cells were treated by 120 μmol/L NCTD for various time periods. *n*=3. Mean±SD.

NCTD-induced morphological changes and DNA fragmentation of HeLa cells The nuclear morphological changes were observed by Hoechst 33258 staining. In control group, HeLa cells were round in shape and stained homogeneously. After 24 h treatment with NCTD, blebbing nuclei and granular apoptotic bodies appeared (Fig 2A, arrow).

Typical DNA fragmentation was observed in NCTD-treated HeLa cells (Fig 2B), which is one of characteristics of apoptosis.

Effect of caspases on NCTD-induced cytotoxicity in Hela cells To investigate the role of caspases in NCTD-induced cell apoptosis, HeLa cells were treated with NTCD 120 μmol/L for 24 h in the absence or presence of various caspase inhibitors: z-VAD-fmk (caspase-family inhibitor 20 μmol/L), z-DEVD-fmk (caspase-3 inhibitor, 20 μmol/L), z-IETD-fmk (caspase-8 inhibitor, 20 μmol/L), Ac-LEHD-CHO (caspase-9 inhibitor, 20 μmol/L), Ac-YVAD-cmk (caspase-1 inhibitor, 20 μmol/L). z-VAD-fmk, z-DEVD-fmk z-IETD-fmk and Ac-LEHD-CHO partially blocked NCTD-induced HeLa cell apoptosis. The inhibitory ratio of cell growth was 24.86 %, 30.44 %, 38.38 %, and 36.40 %, respectively

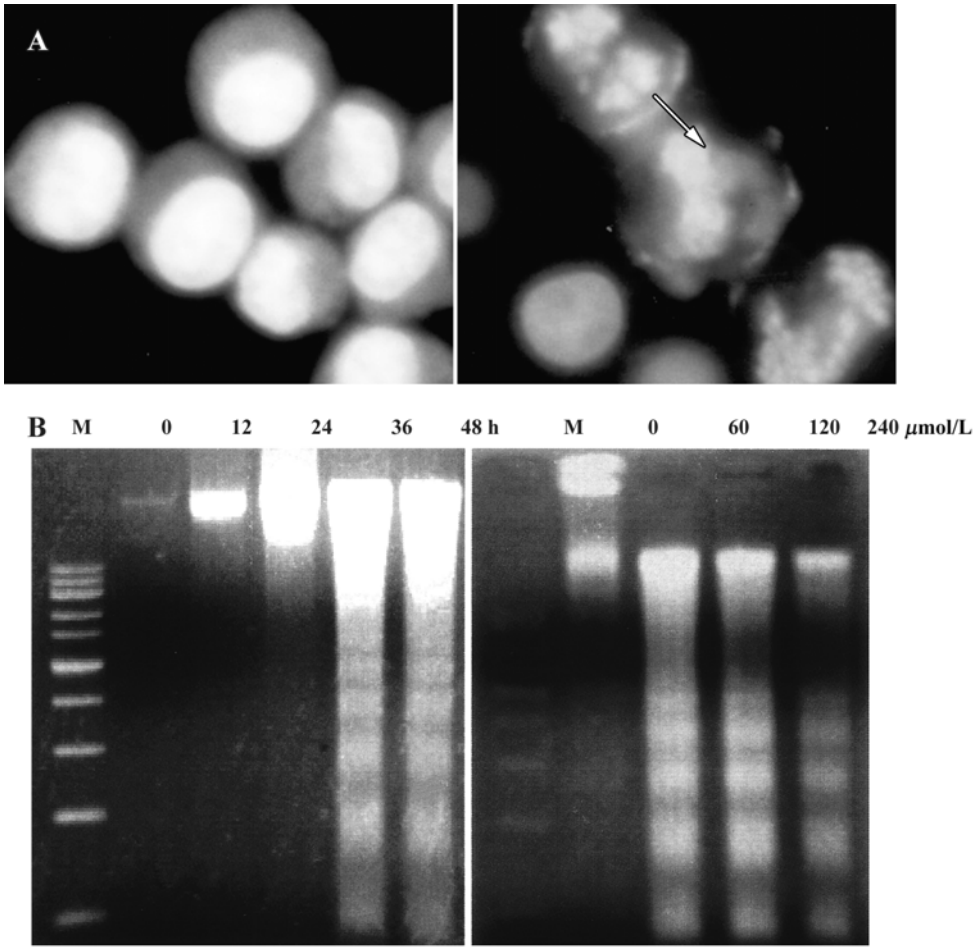

Fig 2. NCTD-induced morphological changes of cell nuclei and DNA fragmentation in HeLa cells. (A) HeLa cells were treated with (right) or without (left) 120 μmol/L NCTD for 24 h (×400, Hoechst 33258 staining). (B) The cells were cultured in the presence of NCTD 120 μmol/L at different time periods (left) or with different concentrations of NCTD for 36 h (right). Lane M: DNA molecular marker.

(Fig 3A). To further examine the participation of caspases in NCTD-induced apoptosis, caspase-3, -8, and -9 activities were measured. Caspase activities were obviously enhanced after the cells were treated with NCTD 120 μmol/L (Fig 3B).

To further examine the effect of caspase-3 on the cell apoptosis, the protein expression of ICAD, the substrate of caspase-3, was examined by Western blot analysis. The ICAD protein degradation was significant after 24 h incubation with NCTD (Fig 3C). The result was accordant to that of caspase activity assay.

Involvement of Bcl-2 family proteins in NCTD-induced HeLa cell apoptosis After incubation with NCTD, expression of Bcl-x_L protein was down-regulated, on the contrary, the level of Bax protein was increased (Fig 4).

Effect of MAPKs on NCTD-induced cytotoxicity in Hela cells The specific inhibitors for ERK (PD98059), JNK (SP600125), and p38 (SB203580) were used to assess functions of three MAPKs in the NCTD-induced HeLa cell death. After 24-h incubation, PD98059 20 μmol/L and SP600125 40 μmol/L partially blocked the cell death induced by NCTD 120 μmol/L, however, SB203580 40 μmol/L had no effect on cell death (Fig 5A). To further confirm these results, the effect of NCTD on protein expression of MAPKs and their phosphorylation were examined. NCTD did not alter p38 expression and the phosphorylation of p38. Unlike p38 MAPK, the protein expression of ERK and JNK were both decreased by NCTD after 36 h incubation. The phosphorylation of ERK was up-regulated at the same time point, but the phosphorylation of JNK was enhanced just after 12 h incubation with NCTD and reached the maximal expression at 36 h. Moreover, after 48 h treatment the increase of phosphorylation of JNK protein expression almost disappeared and the ex-

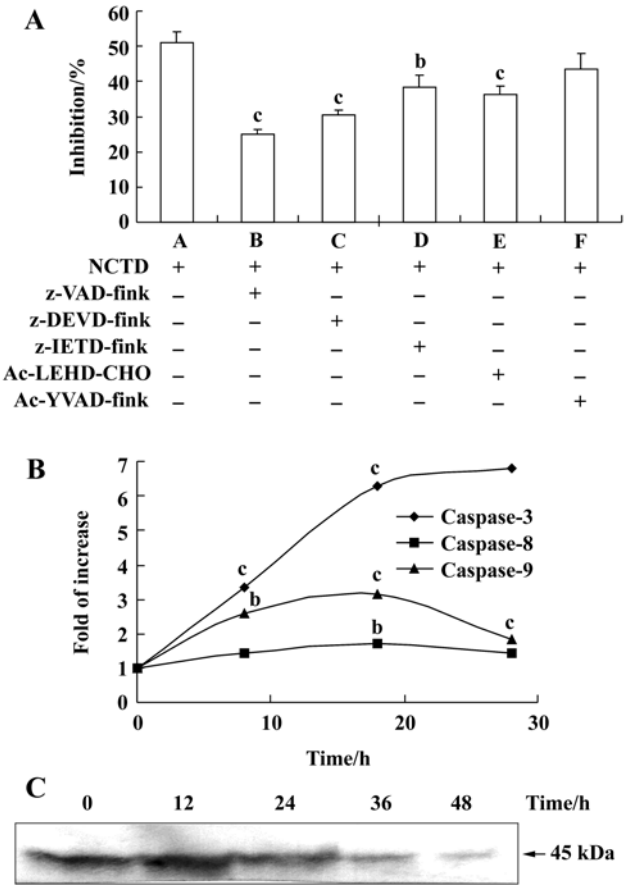

Fig 3. Effect of caspases on NCTD-induced cytotoxicity in Hela cells. (A) Effect of caspase inhibitors on NCTD-induced HeLa cells apoptosis. The cells were cultured in the presence or absence of caspase inhibitors. Two hours prior to the addition of 120 μmol/L NCTD, various caspase inhibitors were added, then further incubated for 24 h. *n*=3. Mean± SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* group A. (B) Activities of caspase-3, -8, and -9 in NCTD-treated HeLa cells were assayed after treatment with NCTD 120 μmol/L for 0, 8, 18, and 28 h. *n*=3. Mean± SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* 0 h. (C) The expression of ICAD in NCTD-treated Hela cells. The cells were incubated with NCTD 120 μmol/L for 0, 12, 24, 36, and 48 h.

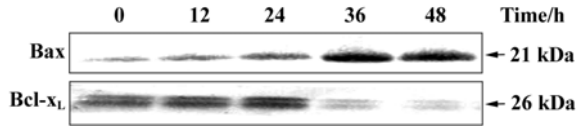

Fig 4. The expression of Bax and Bcl-x_L in NCTD-treated Hela cells. The cells were incubated with 120 μmol/L NCTD for 0, 12, 24, 36, and 48 h.

pression of phosphorylation of ERK protein was further enhanced (Fig 5B). The results suggested that ERK, JNK and p38 had different effects on NCTD-induced

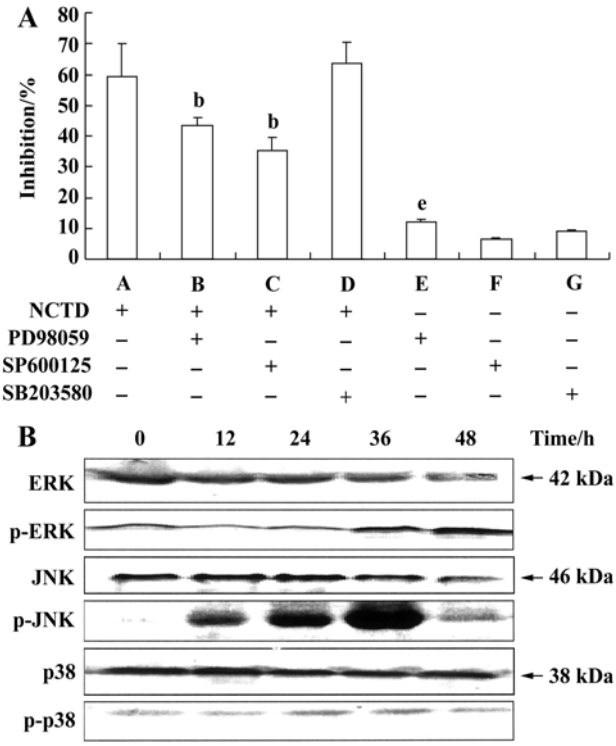

Fig 5. Effect of MAPKs on NCTD-induced cytotoxicity in Hela cells. (A) Effect of MAPK inhibitors on NCTD-induced apoptosis. Two hours prior to the addition of 120 μmol/L NCTD, HeLa cells were treated with MAPK inhibitors, PD98059 (20 μmol/L), SP600125 (40 μmol/L), SB203580 (40 μmol/L), then further incubated for 24 h. *n*=3. Mean±SD. $^{b}P<0.05$ *vs* group A, $^{e}P<0.05$ *vs* untreated group. (B) The expression of MAPK (ERK, JNK, p38) and phosphorylated-MAPK (p- ERK, p-JNK, p-p38) in NCTD-treated Hela cells. The cells were incubated with NCTD 120 μmol/L for 0, 12, 24, 36, and 48 h.

HeLa cell apoptosis.

DISCUSSION

Apoptosis plays a critical role in embryogenesis, carcinogenesis and virally infected cell death[2]. Apoptotic cells, different from necrotic cells, appear to have characteristic changes including membrane blebbing, shrinking and nuclear fragmentation. The end point in apoptosis involves the fragmentation of the cells into membrane-bound vesicles containing remnants of protein and fragmented chromatin, referred to as apoptotic bodies[3,4]. This study showed that NCTD inhibited HeLa cell growth in a time- and dose-dependent manner and induced apoptosis in HeLa cells by morphological observation and DNA fragmentation.

There are at least two major apoptotic pathways, initiated by caspase-8 and caspase-9 respectively, which

can activate caspase cascades. The active executioners, such as caspase-3, promote apoptosis by cleaving cellular substrates (such as ICAD) that induce the morphological and biochemical features of apoptosis[16,17,7]. In this study, caspase-family, -3, -8, and -9 inhibitors (z-VAD-fmk, z-DEVD-fmk, z-IETD-fmk, Ac-LEHD-CHO, respectively) partially blocked NCTD-induced HeLa cell apoptosis. At the same time, the activities of caspase-3, -8, and -9 were up-regulated and the protein expression of ICAD was significantly down-regulated, indicating that caspases participated in this apoptotic process. It has been reported that Bcl-2 and Bcl-x_L, anti-apoptotic protein localized in mitochondria, can be cleaved by caspase-3 and thus be converted to a pro-apoptotic protein similar to Bax. The oligomerization of Bax in the mitochondrial membrane has been shown to induce cytochrom *c* release and the subsequent steps (including caspase-9 and caspase-3) in the execution phase of apoptosis[18,6,10]. HeLa cells treated with NCTD exhibited elevated levels of pro-apoptotic Bax expression, while anti-apoptotic Bcl-x_L was down-regulated after the significant increase of caspase-3 activity. Based on these results we concluded that caspase and mitochondrial protein played important roles in the apoptotic pathway of NCTD-treated HeLa cells. On the other hand, since NCTD-induced HeLa cell apoptosis was only partially reduced by caspase inhibitors, it is possible that other apoptotic pathways may also participate in this process.

The members of MAPKs, including ERK, JNK, and p38, were reported to play important roles in cell proliferation and death. Each MAPK subtype is activated by phosphorylation on threonine and tyrosine residues by upstream dual-specificity kinases. In general, the ERK cascade appears to mediate signals promoting cell proliferation, differentiation or survival, whereas the p38 and JNK cascades appear to be involved in the cell responses to stresses[19]. In our studies, each MAPK of HeLa cells was affected in different ways after NCTD treatment. NCTD had no effects on the protein expression of p38 and the phosphorylation of p38 in HeLa cells, furthermore, p38 inhibitor, (SB203580 40 μmol/L), failed to attenuate the cell death induced by NCTD after 24-h incubation, indicating that p38 MAPK was not responsible for HeLa cell death induced by NCTD. On the contrary, ERK inhibitor (PD98059, 20 μmol/L) and JNK inhibitor (SP600125, 40 μmol/L) inhibited NCTD-induced HeLa cell death after 24-h incubation. Moreover, the expression of ERK and JNK were down-regulated, whereas the phosphorylation of ERK and JNK were up-regulated. However, the phosphorylation of ERK and JNK began to increase at different time points. After 12 h incubation, the protein expression of phosphorylated JNK was significantly enhanced and reached to the maximal peak at 36 h, whereas the up-regulation of phosphorylated ERK protein expression appeared after 36 h incubation. It suggested that ERK and JNK were responsible for NCTD-induced apoptosis in HeLa cells and worked at different time stages. Phosphorylation of JNK might participate in the initiation of apoptosis, whereas phosphorylation of ERK was exerted in the latter stages of NCTD-induced apoptosis. In our study, ERK had no protective effects on NCTD-induced HeLa cell death as reported in PC-12 cell apoptosis induced by nerve growth factor (NGF) withdrawal[12]. These results demonstrate that activation of distinct MAPK subtype is dependent on the cell types and the stimuli.

In conclusion, caspase cascade, ERK and JNK MAPK cascade, and mitochondrial protein, including Bax and Bcl-x_L, were involved in the NCTD-induced HeLa cell apoptosis. The more detailed mechanism of NCTD-induced HeLa cell apoptosis remains to be elucidated.

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