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ORIGINAL ARTICLE

Induction of Apoptosis in Human Hep3B Hepatoma Cells by Norcantharidin through a p53 Independent Pathway via TRAIL/DR5 Signal Transduction*

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ABSTRACT **Objective:** To investigate the inhibitory activities of norcantharidin (NCTD), a demethylated analogue of cantharidin, on Hep3B cells (a human hepatoma cell line) with deficiency of p53. **Methods:** The survival rate of the Hep3B cells after treating with NCTD was measured by MTT assay. Cell cycle of treated cells was analyzed by flow cytometry, and DNA fragmentation was observed by electrophoresis. The influence of inhibitors for various caspases and anti-death receptors antibodies on the NCTD-induced apoptosis in the cells was determined. **Results:** NCTD treatment resulted in growth inhibition of Hep3B cells in a dose- and time-dependent manner. Cell cycle analysis of the cells after treatment with NCTD for 48 h shows that NCTD induced G_2M phase arrest occurs at low concentration (≤25 μmol/L) but G_0G_1 phase arrest at high concentration (50 μmol/L). The addition of both caspase-3 and caspase-10 inhibitors completely inhibited DNA fragmentation. Addition of anti-TRAIL/DR5 antibody significantly inhibited DNA fragmentation. **Conclusion:** NCTD may inhibit the proliferation of Hep3B cells by arresting cell cycle at G_2M or G_0G_1 phase, and induce cells apoptosis via TRAIL/DR5 signal transduction through activation of caspase-3 and caspase-10 by a p53-independent pathway.

KEYWORDS norcantharidin, caspase, apoptosis, death receptors

Mylabris is a dried body of Chinese blister beatle, which has been used in Chinese medicine to treat tumor for many centuries. The antitumor activity was identified to be associated with active ingredient, cantharidin.[1] However, cantharidin also produced nephrotoxic and phlogogenic side effects. Chemical analogs were therefore synthesized to reduce toxicity. Among them, norcantharidin (NCTD), was found less toxic,[1] but retains potent anti-tumor activities. Elegant studies demonstrated that NCTD could inhibit the growth of various tumor cells including HepG2 human hepatoma cells, HL-60 human promyelocytic leukemia cells, K562 human myeloid leukemia cells, RT2 rat glioma cells and U251 human glioblastoma cells.[2-8]

In clinics, NCTD has been widely used in the treatment of cancer, especially hepatoma, in China.[1] It was found that NCTD can induce apoptosis of HepG2 cells, a cell line of human hepatoma cells, through Fas and tumor necrosis factor (TNF) receptor via the activation of caspase cascade by a p53 dependent pathway.[9-12] However, most of the cancer cells derived from primary human hepatoma tissue have mutant p53, which can be a poor prognostic factor.[13] It is necessary to investigate the effect of NCTD on the hepatoma cells with mutant p53 and clarify the mechanism. In this study, we evaluate the inhibitory effect of NCTD on the proliferation of Hep3B cells, a human hepatoma cell line which has lost the function of p53 as a result of deficiency. We further investigate whether NCTD inhibits the growth of Hep3B cells by inducing apoptosis through caspase cascade via death receptors pathway.

METHODS

Cell Line, Reagents and MTT Cell Growth Assay

Hep3B cells, a kind-gift from Dr. Shih-Lan Hsu, were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and penicillin/streptomycin at 37 ℃

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in 5% CO_2 until the cells reached confluency. The cells were then seeded into 96 well microplate at a density of 10^3 cells per well in DMEM medium and left overnight to adhere. Various concentrations (0–100 μmol/L) of NCTD, purchased from Beijing Fourth Pharmaceutical Cooperation, were added to the culture wells. After 24, 48 or 72 h treatment, cell growth was assessed by MTT assay as described previously.[14] The experiments were performed at least three times.

Cell Cycle Analysis

The Hep3B cells were treated with various doses of NCTD for 48 h. Cells were harvested, washed in phosphate buffered saline (PBS) and the cell pellets were fixed in 80% alcohol overnight at 4 ℃. The pellets were suspended in 1 mL of PBS containing 0.5 mg/mL of RNase A and 0.5 mg/mL of propidium iodide (PI) for 1 h in the dark. Cells were analyzed using flow cytometry (Becton Dickinson, USA). Data of cell cycle and subG_1 ratio were analyzed using CellQuest and WinMDI 2.8 software.

Expression of Active Caspase-3

Hep3B was seeded on glass cover slips overnight and treated with NCTD for 48 h and then was fixed in acetone for 10 min at –20 ℃ and washed twice with 0.1% Triton-X100-PBS. The cells were then soaked in 0.3% H_2O_2 for 10 min to removed intracellular peroxidase. After washing with PBS, the cells were blocks with 5% donkey serum for 45 min. The cells were then incubated with rabbit anti-active caspase-3 antibody (Jackson Immuno Research Inc., USA) in 1:125 dilution with 1% donkey serum 1 h and washed with PBS, followed by adding biotinylated donkey anti-rabbit antibody in (1:500 dilution) and HRP-conjugated streptavidin. The cells were washed with PBS in each step and finally were incubated with 3-amino-9-ethylcarbazole (AEC) substrate for 10 min. The cells were observed under a microscope (BX50, Olympus, Tokyo, Japan) and the positive cells with active caspase-3 showed in red color.

DNA Fragmentation Analysis

Hep3B cells were seeded in petri-dishes at a density of 1 x 10^5 cells/mL for overnight at 37 ℃. The 50 μmol/L specific inhibitors of caspase-2 (z-VDVAD-fmk), caspase-3 (z-DEVD- fmk), caspase-8 (z-IETD-fmk), caspase-9 (z-LEHD- fmk), caspase-10 (z-AEVD-fmk, R&D Systems, Minneapolis, MN, USA) were added to the cells and incubated for 1 h. Another set of seeded cells were incubated with 1 μg/mL anti-human DR4/TRAIL-R1, DR5/TRAIL-R2 monoclonal antibodies (R&D Systems) for 1 h. All these treated cells were incubated with 12.5 μmol/L, NCTD and the cell population was harvested by trypsin-ethylenediaminetetraacelic acid (EDTA) and washed once with PBS. The pellet after centrifuging was suspended in 10 mmol/L EDTA-PBS and 5 mol/L NaCl was added. Then lysis buffer [0.6% sodium dodecl sulfate (SDS), 10 mmol/L EDTA, pH7.0] was added, mixed well, and stored overnight at 4 ℃. After extracting of DNA using phenol-chloroform method, RNase A (1 mg/mL) was added into the pellet at 37 ℃ and incubated for 10 min. DNA extract was then electrophoresed in 1.2% agarose gel with ethidium bromide. DNA fragment bands were examined using a UV transluminator.

Western Blotting

Cell pellets (1×10^6 cells) were washed with PBS, lysed by CytoBuster protein extraction reagent (Novagen) for 30 min at 4 ℃, and then centrifuged for 10 min at 10,000 *g*. Equal amounts of supernatant were run in 10% SDS-PAGE gel and transferred onto nitrocellulose membranes under semi-dry status. Nitrocellulose blots were blocked with 3% non-fat dry milk in phosphate buffer saline tween (PBST) containing 0.1%Tween 20 and incubated at room temperature for 2 h with primary antibody such as mouse monoclonal anti-human DR4, mouse monoclonal anti-human DR5 (both from R&D Systems), and mouse monoclonal anti-human actin (Santa Cruz Biotechnology, Santa Cruz, Ca, USA) as an internal control. The membranes were washed with PBS and incubated with goat anti-mouse IgG-horseradish peroxidase (HRP) secondary antibody for 30 min. Substrates were visualized using enhanced chemiluminescense (Perkin Elmer, Shelton, CT, USA) and analyzed with Fusiom SL2 Image station (Vilber Lourmat, France) and protein quantitation with Bio-1D software.

Statistical Analysis

Data are expressed as means ± standard deviation. Statistical analysis was done using one way ANOVA (SPSS version 14.0). A P value <0.05 was considered significant.

RESULTS

Suppression of NCTD on the Proliferation of Hepatoma Cell Lines *in vitro*

As shown in Figure 1, the effect of NCTD on

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cell growth of Hep3B cells was examined at various concentrations between 1.6 and 100 μmol/L for 24, 48 and 72 h. Proliferation of Hep3B cells was markedly inhibited by NCTD treatment in a dose- and time-dependent manner. There was a significant inhibition at concentrations of 25–100 μmol/L NCTD. The half maximal inhibitory concentration (IC_{50}) of NCTD on Hep3B cells after culture for 24, 48 and 72 h was 41.9, 22.1 and 19.25 μmol/L, respectively.

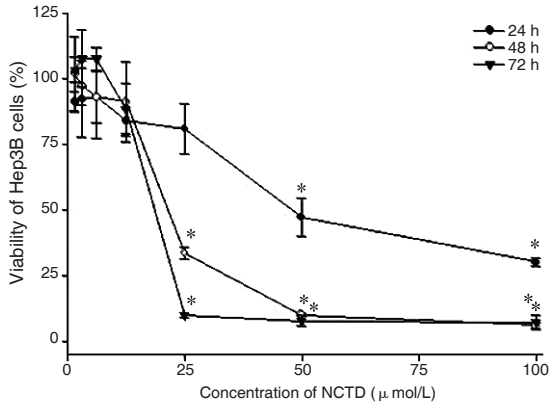

Figure 1. Cell Growth Inhibition by NCTD in Hep3B Cells

Notes: Hep3B cells were treated with different concentrations (0–100 μmol/L) of NCTD for 24, 48 and 72 h. After treatment, cells were collected and the percentage of surviving cells were determined by MTT assay. The results were expressed as the mean value of triplicate samples and the standard deviation (bars). $^{*}P<0.001$, compared with control

Cell Cycle Arrest of Hep3B Cells after NCTD Treatment

To investigate whether NCTD induces cell cycle arrest in Hep3B cells, the nucleus of cells stained with the DNA dye, PI, were analyzed by flow cytometry after treatment with varying concentrations of NCTD for 48 h. As shown in Figure 2 and Table 1, PI stained DNA content increased in G_2M phase of cell cycle after treatment with low concentrations of NCTD (25 μmol/L) without a dose-dependent manner. However, it appeared as a peak of $subG_1$ phase after treatment with 50 μmol/L of NCTD. Most of Hep3B cells died after treatment with 100 μmol/L of NCTD and hence cell population could not be detected.

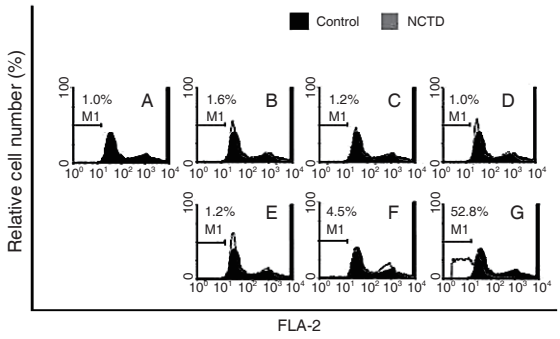

Figure 2. Influence of Different Concentrations of NCTD on Cell Cycle Profile of Hep3B Cell Line

Notes: Hep3B cells were treated with NCTD under concentrations of 0 (A), 1.6 (B), 3.1 (C), 6.3 (D), 12.5 (E), 25 (F) and 50 (G) μmol/L, respectively, for 48 h. The cell cycle profiles were analyzed by flow cytometry with PI staining. Figures were obtained from three separated experiments with similar patterns

DNA Fragmentation in Hep3B Cells Induced by NCTD

DNA fragmentation of treated cells was analyzed by gel electrophoresis to determine apoptosis in Hep3B cells after being treated with or without NCTD for 24 to 72 h. As shown in Figure 3, a phenomenon of DNA cleavage into DNA fragments, a typical feature of apoptosis, was observed in Hep3B cells after NCTD treatment. Time course study in Hep3B cells showed that the formation of DNA fragments appeared earlier at 24 h under treatment with NCTD at a high concentration of 100 μmol/L, but appeared at 72 h under treatment with NCTD at a low concentration of 1.6 μmol/L.

Detection of Active Caspase-3

Figure 4 shows the appearance of red micronucleation staining in apoptotic cells after immunostaining with active caspase-3. Large number of micronucleation staining are observed in cells treated with NCTD in 50 μmol/L dose for 48 h.

Table 1. The Relative Distribution of Hep3B Cells in Cell Cycles after Being Treated with Various Concentrations of NCTD ($\bar{x} \pm s$)

Cell cycle	*n*	Relative distribution (%)						
		NCTD 0	1.6	3.1	6.3	12.5	25	50 (μmol/L)
$subG_1$	3	2.2 ± 3.1	2.7 ± 1.5	2.1 ± 2.1	2.4 ± 1.2	1.8 ± 2.2	5.5 ± 2.3*	56.3 ± 4.1**
G_0G_1	3	58.7 ± 3.1	57.7 ± 4.5	56.6 ± 5.2	54.3 ± 6.7	53.9 ± 6.1	66.5 ± 30.1	82.0 ± 31.2
G_2M	3	0.1 ± 0.1	1.5 ± 2.6	1.5 ± 2.6	2.1 ± 3.1	2.6 ± 3.8	2.0 ± 3.5	0

Notes: $^{*}P<0.05$, $^{**}P<0.01$, compared with control

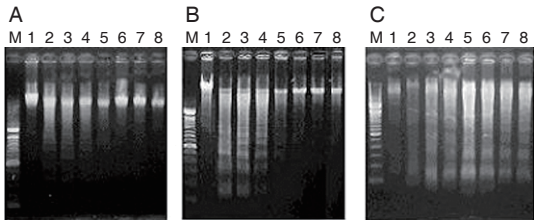

Figure 3. Induction of DNA Fragmentation by NCTD

Notes: Hep3B cells were treated with different concentrations of NCTD for 24 (A), 48 (B) and 72 (C) h. The extracted DNA of cultured cells was further analyzed by electrophoresis. M: 100 bp ladder; lane 1: blank; lane 2: 100 μmol/L; lane 3: 50 μmol/L; lane 4: 25 μmol/L; lane 5: 12.5 μmol/L; lane 6: 6.3 μmol/L; lane 7: 3.1 μmol/L; lane 8: 1.6 μmol/L. The figures were obtained from three independent experiments with similar patterns

The Influence of Inhibitors of Caspase 3 and Caspase 10 on Apoptosis of Hep3B Cells Induced by NCTD

To determine whether caspase cascade is involved in the apoptosis of Hep3B cells induced by NCTD, Hep3B cells were incubated 50 μmol/L concentration of specific inhibitors of caspase for 1 h and then further cultured with 12.5 μmol/L of NCTD for 48 h. As shown in Figure 5, pretreatment with the inhibitor of caspase-3 (z-DEVD- fmk) or caspase-10 (z-AEVD-fmk) markedly inhibited NCTD-induced DNA fragmentation of Hep3B cells. However, pretreatment with the inhibitor of caspase-2 (z-VDVAD-fmk), caspase-8 (z-IETD-fmk) or caspase-9 (z-LEHD-fmk) did not prevent NCTD-induced apoptosis of Hep3B cells.

The Influence of Anti-DR4/TRAIL-R1 or Anti-DR5/TRAIL-R2 Antibody on NCTD-Induced Hep3B Cells Apoptosis

To further confirm the role of death receptors in Hep3B cells apoptosis induced by NCTD, we incubated Hep3B cells with or without 1 μg/mL neutralizing antibody to DR4/TRAIL-R1 or DR5/TRAIL-R2 for 1 h before treatment with 25 μmol/L of NCTD for 48 h. As shown in Figure 6, incubation of anti-human DR5/TRAIL-R2 monoclonal antibodies but not anti-DR4/TRAIL-R1 antibodies markedly inhibited

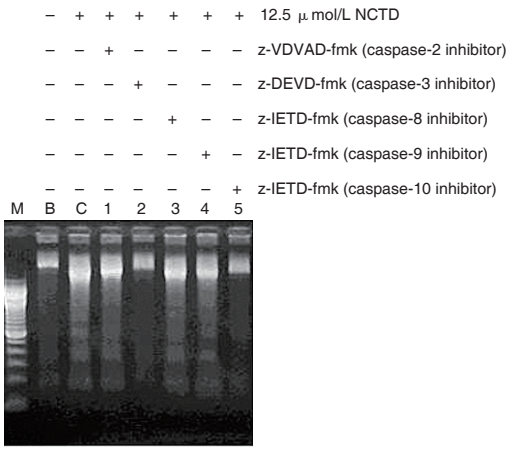

Figure 5. Influence of Caspase Inhibitors on Apoptosis of Hep3B Cells Induced by NCTD

Notes: Hep3B cells were pretreated with 50 μmol/L of specific inhibitors of caspase-2 (lane 1), caspase-3 (lane 2), caspase-8 (lane 3), caspase-9 (lane 4) and caspase-10 (lane 5), respectively for 1 h. The cells were further treated with 12.5 μmol/L of NCTD for 48 h. The DNA of cultured cells was analyzed by electrophoresis. M: 100 bp ladder; B: cells, as blank, without treatment with any inhibitor of caspase and NCTD; C: cells, as control, treated with only NCTD but not any inhibitor of caspase. The figure was obtained from three times repeated

DNA fragmentation induced by NCTD.

Expression of DR4/TRAIL-R1 and DR5/TRAIL-R2 Proteins in Hep3B Cells after Treatment with NCTD

To confirm whether DR4/TRAIL-R1 and DR5/TRAIL-R2 are involved in NCTD-induced Hep3B cells apoptosis, DR4/TRAIL-R1 and DR5/TRAIL-R2 proteins were detected by Western blot analysis in Hep3B cells after treatment with NCTD for 48 h. Both DR4/TRAIL-R1 and DR5/TRAIL-R2 proteins were seen expressed in Hep3B cells after treatment with NCTD (Figure 7). The protein expression in fold changes is shown in Figure 8. The expression of DR4 in the cells after treatment with various concentrations of NCTD is not showing a significant change when compared to the control. The expression of DR5 is seen increased from 1.1 to 1.4 folds on treatment with various concentrations of NCTD.

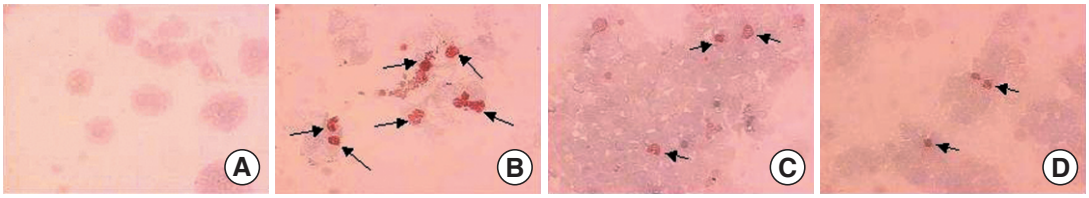

Figure 4. Immunohistochemical Stainning of Active Caspase-3 in Hep3B Cells after Treatment of NCTD for 48 h

Notes: A: blank, B: NCTD 50 μmol/L,C: 25 μmol/L, D:12.5 μmol/L. After treatment, cells were fixed and stained with anti-active caspase-3 antibody. Arrows indicate active caspase-3 positive cells (×100). Figures were representative of three independent experiments

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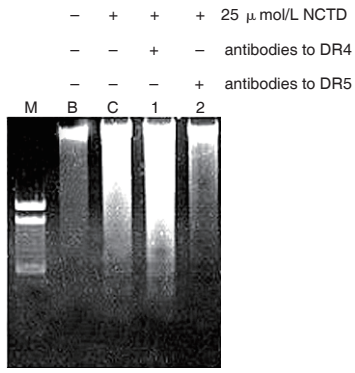

Figure 6. The Influence of Neutralizing Antibody to DR4/TRAIL-R1 or DR5/TRAIL-R2 on NCTD-Induced Hep3B Cells Apoptosis

Notes: Cells were treated with or without 1 μg/mL of neutralizing antibody to DR4/TRAIL-R1 (lane 1) or DR5/TRAIL-R2 (lane 2) for 1 h, and then treated with (C) or without 25 μmol/L of NCTD for 48 h. The DNA of cultured cells was analyzed by electrophoresis. M: 100 bp ladder; B: cells, as blank. The figure was representative of three independent experiments with similar patterns.

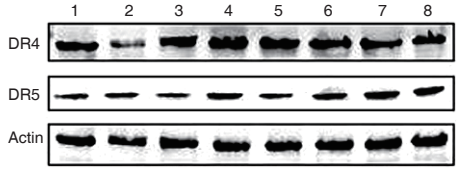

Figure 7. Effect of NCTD on the Expression of DR4/TRAIL-R1 and DR5/TRAIL-R2 Proteins in Hep3B Cells

Notes: Hep3B cells were treated with 0 (lane 1), 100 (lane 2), 50 (lane 3), 25 (lane 4), 12.5 (lane 5), 6.3 (lane 6), 3.1 (lane 7) and 1.6 (lane 8) μmol/L of NCTD for 48 h. The lysates of cultured cells were analyzed by Western blot, using actin as an internal control. Figures were representative of three independent experiments

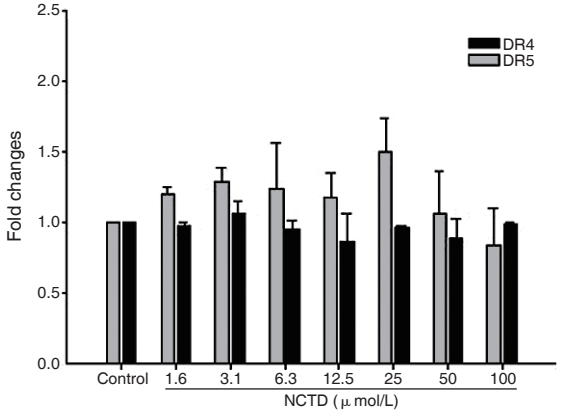

Figure 8. The Fold Change of the Protein Expressions

Notes: All data were expressed as mean ± SD from three independent experiments

DISCUSSION

NCTD, a synthetic analogue of cantharidin, has been widely used in the treatment of different kinds of cancers in China, especially in the treatment of patients with primary hepatoma. In the present study, NCTD is found to be able to inhibit the proliferation of Hep3B cells from a concentration equal to or greater than 12.5 μmol/L. Our results suggest that NCTD can inhibit the proliferation of Hep3B cells by a dose- and time-dependent manner. These results are consistent with a previous study reported by Chen, et al[3] that NCTD has a strongly inhibitory activity on the cell growth of Hep3B cells.

The cell cycle analysis indicates that DNA content in Hep3B cells is increased in G_2M phase of cell cycle under treatment with low concentrations of NCTD (1.6–25 μmol/L) without a dose-dependant manner but appeared as a peak in G_0G_1 phase of cell cycle under treatment with 50 μmol/L of NCTD. These results suggest that, NCTD may induce cell cycle arrest of Hep3B cells in G_2M phase at low concentrations and in G_0G_1 phase at high concentrations. This result is also similar to the reports of other investigations that NCTD can induce cell cycle arrest at G_2M phase in glioblastoma cells and colorectal carcinoma.[6,15] A peak of subG_1 phase after treatment with 50 μmol/L of NCTD indicated apoptotic event induced by NCTD in Hep3B.

Many *in vitro* studies reported that NCTD can induce apoptotic effect on various tumor cell lines,[3,6-8] but the mechanism remains unclear. In this study, DNA fragmentation is detected as early as 24 h in extremely high concentration of NCTD (100 μmol/L) and could be detected in low concentration of NCTD by 72 h. These results suggest that, NCTD can induce apoptosis of Hep3B cells early by high concentration and lately by low concentrations.

The Hep3B cells treated with NCTD shows strong active caspase-3 expression on immunostaining. With the increase of treatment dose and time, the activation of caspase-3 became more marked, paralleled with apoptosis phenomenon. These results reveals that NCTD may induce apoptosis through caspase cascade activation, followed by DNA cleavage into 180–220 bp fragments.

Many reports suggest that, activation of caspase system may induce cell apoptosis.[16,17] In the present study, pretreatment with inhibitors of caspase-3 or caspase-10 could nearly completely suppress DNA fragmentation phenomena of Hep3B cells treated with

NCTD, while inhibitors of caspase-2, caspase-8, or caspase-9 cannot. These results suggest that NCTD may induce apoptosis mostly through the activation of caspase-3 and caspase-10.

A number of anti-tumor agents can cause initiation of apoptosis through different death receptors. The death receptor pathway is one of the major biochemical pathways leading to the induction of apoptosis. Apoptosis initiates when extra-cellular death signal proteins [FasL, TNF α and TNF-related apoptosis-inducing ligand (TRAIL)] bind to their cognate death receptors including Fas, TNFR1, TRAIL-R1/DR4 and TRAIL-R2/DR5.[9,12] The death receptor complexes or a mitochondrion-dependent mechanism may cause caspases activation.[18] In this study we found that, both TRAIL-R1/DR4 and TRAIL-R2/DR5 proteins are expressed in Hep3B cells after NCTD treatment. However, only neutralizing antibody to TRAIL-R2/DR5 but not neutralizing antibody to TRAIL-R1/DR4 can block the apoptotic event in Hep3B cells induced by NCTD. These results indicates that, TRAIL-R2/DR5 but not TRAIL-R1/DR4 may be involved in the pathway of NCTD induced Hep3B cells apoptosis. TRAIL-R2/DR5 is one of the TRAIL receptors that contains cytoplasmic death domains and preferentially induces apoptosis in tumor cell lines but not in normal cells.[19] TRAIL can induce apoptosis via a caspase signaling cascade that executes apoptosis independently of the pro-apoptotic machinery of mitochondria.[20] Interestingly, TRAIL selectively binds to TRAIL-R1/DR4 or TRAIL-R2/DR5 of tumor cells without association with systemic toxicity in animal model.[21] These advantages are preferable for chemotherapy.

Except TRAIL receptors, other death receptors such as Fas and TNF receptor also may be involved in the NCTD-induced apoptosis of Hep3B cells. However, Hep3B is found to be lacking Fas receptor.[22,23] Therefore, it is unlikely that Fas receptor is involved in the process of apoptosis of Hep3B cells induced by NCTD. Although TNF receptor is found to be expressed in Hep3B cells,[24] in another experiment we did not found the expression of TNF receptor in Hep3B cells after treatment with NCTD (data not shown). This fact taken together with the result that the addition of neutralizing antibody to TRAIL-R2/DR5 nearly completely inhibited the apoptosis of Hep3B cells suggests that NCTD may induce apoptosis of Hep3B cells mostly via TRAIL-R2/DR5 pathway.

Hong, et al[6] reported that NCTD induced apoptosis is p53-dependent. Chen, et al[3] and Kok, et al[25] observed that in hepatoma and oral cancer cells, apoptosis induced by NCTD occurred in different status of p53. This implies that p53 may play an important role in gene regulation, but apoptosis ocurred independent of p53. In this study, NCTD is found to be able to inhibit the proliferation of Hep3B cells, a p53 and Fas deficient human cell line, with cell cycle arrest in G_2M phase or G_0G_1 phase. Apoptosis phenomenon is found in Hep3B cells as early as 24 h in high concentrations and lately by 72 h in low concentrations of NCTD. The apoptosis of Hep3B cells induced by NCTD could be inhibited by the inhibitors of caspase-3 and caspase-10 but not inhibitors of caspase-2, caspase-8, or caspase-9. Addition of neutralizing antibody to TRAIL-R2/DR5 but not TRAIL-R1/DR4 nearly completely inhibited the NCTD-induced apoptosis in Hep3B cells. The above results suggest that NCTD may induce apoptosis via TRAIL-R2/DR5 through the activation of caspase-3 and caspase-10 by a p53-independent manner.

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