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Norcantharidin-induced apoptosis is *via* the extracellular signal-regulated kinase and c-Jun-NH_2-terminal kinase signaling pathways in human hepatoma HepG2 cells

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1 Norcantharidin (NCTD) is an anticancer drug routinely used against hepatoma in China. Previously, we reported that NCTD could induce mitotic arrest and apoptosis in human hepatoma HepG2 cells. However, the intracellular signaling pathways involved in NCTD-induced apoptotic cell death are still obscure.

2 Caspase inhibitors were used to clarify the role of specific caspase in NCTD-triggered apoptotic process. Results showed that activation of caspase-9/caspase-3 cascade is required for NCTD-induced apoptotic death.

3 To decipher the upstream signals for NCTD-induced apoptosis, we characterized the involvement of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and $p38^{MAPK}$. The role of their downstream targets, transcription factors activating protein-1 (AP-1), and nuclear factor κB (NF-κB) in NCTD-induced apoptosis was also analyzed.

4 Immunoblot analyses and *in vitro* kinase assay demonstrated that NCTD-induced apoptosis was accompanied by the elevations of the levels of phosphorylated form and kinase activity of ERK and JNK, but not $p38^{MAPK}$.

5 The inhibitor of ERK pathway (U0126 or PD98059) or JNK pathway (SP600125) markedly prevented kinase activation, and also greatly reduced NCTD-induced apoptotic cell death.

6 Increased DNA-binding activity of AP-1 and NF-κB was also observed after NCTD treatment.

7 Inhibition of NF-κB activation by PDTC or inhibition of AP-1 activation by curcumin drastically blocked NCTD-induced cell death.

8 These results imply that activation of ERK and JNK, and modulation of downstream transcription factors NF-κB and AP-1, may be involved in NCTD-induced apoptosis.
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Abbreviations: AP-1, activating protein-1; ERK, extracellular signal-regulated kinase; GST-c-Jun, glutathione-*S*-transferase-c-Jun; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; NCTD, norcantharidin; NF-κB, nuclear factor κB; PDTC, ammonium pyrrolidinedinedithiocarbamate; TUNEL, terminal transferase-mediated dUTP-fluorescein nick endlabeling

Introduction

Norcantharidin (NCTD), a demethylated analogue of cantharidin, has been used for the treatment of primary hepatoma, hepatitis, carcinomas of esophagus and gastric cardia, and leukopenia in China (Wang, 1989; Eang *et al*., 1993). Previous studies have shown that NCTD exhibited remarkable inhibitory effect on protein phosphatases 1 and 2A (Mccluskey *et al*., 2001), stimulated granulopoisis in both normal and irradiated mice *in vitro* (Yi *et al*., 1988; 1989), decreased the *N*-acetyltransferase activity in human hepatoma HepG2 cells (Wu *et al*., 2001), and inhibited the proliferation of some cancer cells *in vitro* by retarding progression through the cell cycle (Yang *et al*., 1997; Hong *et al*., 2000; Chen *et al*., 2002). Yang *et al*. (1997) demonstrated that NCTD significantly inhibited the growth of human hepatoma HepG2 cell-transplanted tumor in nude mice, and prolonged host survival. It has been reported that NCTD induced a p53-dependent apoptotic pathway (Hong *et al*., 2000). A recent study showed that NCTD mediated a Fas-dependent apoptotic cell death in human colorectal carcinoma cells (Peng *et al*., 2002). Our previous findings demonstrated that NCTD-induced apoptosis was triggered at the mitotic phase in human hepatoma cells (Chen *et al*., 2002). However, the signaling pathways

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responsible for cell death following NCTD administration are still unclear. Thus, it is important to elucidate the components of certain signal transduction pathways, and the mechanisms that govern the transmission of information.

An intracellular signaling pathway that has recently been implicated in the regulation of apoptosis is the mitogen-activated protein kinase (MAPK) cascade (Whitmarsh & Davis, 1996; Hidenori, 1999). The MAPK family comprises serine/threonine kinases, and consists of three subtypes including the extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and $p38^{MAPK}$. In mammalian cells, MAPKs can transduce diverse extracellular stimuli (including mitogenic growth factors, hormones, cytokines, environmental stresses, and proapoptotic agents) to the nucleus *via* kinase cascades, to regulate proliferation, differentiation, and apoptosis (English *et al*., 1999). ERK is mainly activated by growth factors, and has been shown to be associated with cell proliferation and differentiation (Whitmarsh & Davis, 1996; English *et al*., 1999; Hidenori, 1999). However, this is not always the case, and growing evidence suggests that activation of ERK also contributes to cell death (Julio *et al*., 2001; Seo *et al*., 2001; Yu *et al*., 2001). In contrast, JNK and $p38^{MAPK}$ mediate cellular responses to environmental stresses such as proinflammatory cytokines, UV light, γ-irradiation, growth factor withdrawal, ceramide, protein synthesis inhibitor, heat shock, osmotic shock, and shear stress (Whitmarsh & Davis, 1996; English *et al*., 1999; Hidenori, 1999), and have often been demonstrated to be involved in cell death in many cell types (Chen *et al*., 1998; Yu *et al*., 2001; Engedal *et al*., 2002). In addition, JNK activation is required for apoptotic signaling induced by DNA-damaging agents (Zanke *et al*., 1996) and anticancer drugs (Hung *et al*., 1999; Shtil *et al*., 1999).

The substrates of MAPKs that have been recognized thus far are equally varied, and include metabolic enzymes, cytoskeletal proteins, other signaling molecules, and nuclear transcription factors (Whitmarsh & Davis, 1996; English *et al*., 1999; Hidenori, 1999). Accumulating evidence indicated that the transcription factors such as activating protein-1 (AP-1) and nuclear factor κB (NF-κB) are the downstream targets of MAPK pathway, may either be associated or necessary for induction of apoptosis, and that they are known to control the expression of genes associated with apoptosis (Mercurio & Manning, 1999; Shaulian & Karin, 2002). AP-1 complexes are composed of members of the Jun and Fos families that exist as either homo- or heterodimers. MAPK signaling influences AP-1 activation both by increasing the abundance of AP-1 components and by stimulating their activity (Shaulian & Karin, 2001; 2002). It has been demonstrated that AP-1 transcription factors are involved in both the induction and prevention of apoptosis, depending on cell type and stimuli (Shaulian & Karin, 2001; 2002). NF-κB proteins are members of a family of ubiquitous transcription factors that exist as homo- or heterodimers. NF-κB exists in the cytoplasm in an inactive form associated with inhibitory proteins IκB (Mercurio & Manning, 1999; Chen *et al*., 2001). Activation of the NF-κB-signal cascade results in proteolytic degradation of IκB; this process exposes the nuclear localization signal of NF-κB, thereby allowing the translocation of NF-κB to the nucleus, where it binds its target genes to induce transcription (Mercurio & Manning, 1999; Chen *et al*., 2001). Numerous evidence indicated that NF-κB is involved in regulating apoptosis (Foo & Nolan, 1999; Mercurio & Manning, 1999; Chen *et al*., 2001). Stimulation of NF-κB in response to various stimuli, including viral infection, UV, cytokine TNF, and IL-1 serves to block the process of apoptosis (Foo & Nolan, 1999; Mercurio & Manning, 1999; Chen *et al*., 2001). However, there is also evidence to suggest that NF-κB prevents oncogenesis and promotes apoptosis (Foo & Nolan, 1999). Whether NF-κB promotes or inhibits apoptosis also appears to depend on the specific cell type and the inducers.

Given the widespread involvement of MAPK signaling and their downstream targets, nuclear transcription factors AP-1 and NF-κB, in cell death pathways, the objective of this study was to examine whether the members of MAPK family play crucial roles in NCTD-induced cell death, and whether their downstream targets, the transcription factors NF-κB and AP-1, would contribute to the process. Our findings indicated that ERK, JNK, NF-κB, and AP-1 were activated by NCTD, and these signal transduction pathways are likely involved in the NCTD-induced apoptotic action in human hepatoma HepG2 cells.

Methods

Material

NCTD was synthesized and kindly provided by G.-S. Wang (Beijing Fourth Pharmaceutical Works) Beijing, China. Anti-caspase-3, anti-PARP, anti-FasL, anti-DR3, anti-DR4, anti-TNFRI, and anti-TNFRII antibodies were obtained from PharMingen (San Diego, CA, U.S.A.). Anti-Fas antibody was purchased from Transduction Laboratory, Lexington, KY, U.S.A. Anti-ERK1/2, anti-ERK1/2 phospho-specific, anti-JNK, anti-JNK phospho-specific, anti-$p38^{MAPK}$, anti-$p38^{MAPK}$ phospho-specific, anti-c-Myc and anti-c-Jun antibodies, anthrax[1,9-cd]pyrazol-6(2*H*)-one (SP600125), and glutathione-*S*-transferase-c-Jun (GST-c-Jun) peptides were purchased from Calbiochem Chemical Company (CN Biosciences Notts, U.K.). Anti-phospho-c-Myc and anti-phospho-c-Jun antibodies, and 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) (U0126) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, U.S.A.). 2-[2-Amino-3-methoxy phenyl]-4*H*-1-benzopyran-4-one (PD98059), ammonium pyrrolidinedinedithiocarbamate (PDTC), 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (curcumin), and myelin basic protein (MBP) were obtained from Sigma (St Louis, MO, U.S.A.). The inhibitors of caspase-3 (Z-DEVD-FMK), caspase-8 (Z-IETD-FMK), and caspase-9 (Z-LEHD-FMK) were obtained from Kamiya (Thousand Oaks, CA, U.S.A.).

Cell culture and apoptotic cell determination

Human hepatoma HepG2 cells were maintained in Dulbecco's modified Eagle's medium (Gibco/BRL, MD, U.S.A.), supplemented with 10% fetal bovine serum (HyClone, Utah, U.S.A.), 2 mM glutamine, and antibiotics (100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin), at 37°C in a humidified atmosphere of 5% of CO_2. The medium was changed every 2 days. Apoptotic cells were determined as described below. Cells were seeded at a density of 1×10^5 cells per well onto a 12-well −plate, 24 h prior to drug treatment. NCTD was added to the medium at various concentrations and times.

After incubation, apoptotic cells were measured by *in situ* terminal transferase-mediated dUTP-fluorescein nick end-labeling (TUNEL) assay (Boehringer Mannheim; Roche Applied Science). For *in situ* TUNEL assay, cells were fixed in 2% paraformaldehyde at room temperature for 30 min, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline solution (PBS), and then exposed to terminal transferase reaction mixture (34 mU ml^{-1} terminal transferase, 280 pmol of dATP, 90 pmol of fluorescein-11 dUTP, 30 mM Tris-HCl, 140 mM sodium cacodylate, 1 mM $CoCl_2$, pH 7.2) for 1 h at 37°C in the dark. Cells were subsequently washed with PBS and examined under a fluorescence microscope.

Caspase activity assay

Caspase activity was measured according to the manufacturer's protocol (R&D SYSTEMS). Briefly, cell lysates (100 μg total protein) were added to reaction mixtures (final volume 50 μl) containing fluorogenic substrate peptides specific for caspase-1 (YVAD-AFC), caspase-3 (DEVD-AFC), caspase-8 (IETD-AFC), and caspase-9 (LEHD-AFC). The reaction was performed at 37°C for 2 h. Fluorescence was measured with a fluorescence microplate reader (Thermo Labsystem, Finland; excitation wavelength 400 nm, emission wavelength 505 nm).

Immunoblot analysis

To prepare proteins for immunoblotting, untreated or NCTD-treated cells were lysed in protein lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol, 1% NP-40, 0.3% deoxycholate, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} soybean trypsin inhibitor, 10 μg ml^{-1} leupeptin, 0.5 mM phenylmethylsulfonyl fluoride), and protein concentration was determined using the Bradford method. Equal amounts of sample lysate were separated by sodium dodecyl polyacrylamide gel electrophoresis (SDS–PAGE), and electrophoretically transferred onto PVDF membrane (Millipore). The membrane was blocked with 5% nonfat milk in TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20), and incubated overnight at 4°C with specific primary antibodies, including anti-human caspase-3, poly (ADP-ribose) polymerase (PARP), Fas, FasL, DR3, DR4, TNFRI, TNFRII, ERK1/2, phospho-ERK1/2, JNK, phospho-JNK, $p38^{MAPK}$, phospho-$p38^{MAPK}$, c-Myc, phospho-c-Myc, c-Jun, and phospho-c-Jun antibodies. Subsequently, the membrane was washed with TBST buffer and incubated with the appropriate secondary antibody (horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG). Determinations were performed using enhanced chemiluminescence kits (Amersham; ECL kits).

MAPK kinase activity assay

Cell lysates (300 μg total protein) were immunoprecipitated with anti-human ERK or JNK antibody in the presence of 20 μl of protein A–sepharose beads, then rotated for 4 h at 4°C and washed twice with kinase buffer. ERK and JNK kinase activity was determined by incubation of the immunocomplex in 30 μl of kinase reaction buffer containing 20 mM Tris-HCl, pH 7.5, 5 mM EGTA, 20 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 μg MBP (substrate of ERK) or GST-c-Jun (substrate of JNK) peptides, 5 μCi (γ-^{32}P)ATP (6000 Ci $mmol^{-1}$; DuPont-NEN, Boston, MA, U.S.A.), and 1 μM ATP for 15 min at 37°C. The reaction was stopped by adding 10 μl of 4× Laemmli sample buffer, followed by boiling for 10 min. Samples were electrophoresed on 12% SDS–PAGE and then transferred to the PVDF membrane. Incorporation of ^{32}P was visualized by autoradiography, and quantitated with a PhosphoImager (Molecular Dynamic, Sunnyvale, CA, U.S.A.).

Electrophoretic mobility shift assay

The DNA-binding assay for detection of activated AP-1 and NF-κB was performed as described elsewhere (Berger *et al*., 1993; Rodgers *et al*., 2000). Nuclear extracts were prepared from HepG2 cells after treatment with or without 15 μg ml^{-1} NCTD for the indicated time periods. For electrophoretic mobility shift assay, specific oligonucleotides containing consensus DNA-binding site for AP-1 (5′-biotin-CGCTTGATGAGTCAGCCGGAA-3′ and 5′-biotin-TTCCGGCTGACTC ATCAAGCG-3′) and NF-κB (5′-biotin-AGTTGAGGGGACTTTCCCAGGC-3′ and 5′-biotin-GCCTGGGAAAGTCCCCTCAACT-3′) were used. The single-stranded sense and antisense oligonucleotides were boiled and annealed to generate a double-stranded oligonucleotide. DNA binding was performed at 30°C for 20 min in a final volume of 20 μl, which contained 5 μg of nuclear extract, 5 pmol biotin-labeled NF-κB or AP-1-specific consensus oligonucleotide, 20 μg poly(dI/dC) (Pharmacia, Freiburg, Germany), 2 μl buffer A (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP-40, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride), and 4 μl buffer B (20% Ficoll 400 (Pharmacia, Freiburg, Germany), 100 mM HEPES, pH 7.9, 300 mM KCl, 10 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride). The DNA–protein complexes were separated in a 6% nondenaturing polyacrylamide gel in 0.5× Tris-borate-EDTA buffer (TBE) at 12°C. After running, the gel was soaked with 0.5× TBE buffer for 10 min. Then, the gel was transferred onto a nitrocellulose membrane for 1 h. Subsequently, the membrane was fixed with UV light and blocked with 5% nonfat milk for 30 min. Streptavidin–horseradish peroxidase conjugate was added at 4°C overnight. The activation of AP-1 and NF-κB was detected using Supersignal Chemiluminescent substrate kits (Pierce, Rockford, U.S.A.).

Statistical analysis

In this study, DMSO (0.1%). was used as a solvent vehicle control. All data are presented as means ± s.d. of 12 replicates from four separate experiments. Statistical differences were calculated using Student's *t*-test, with the following significance levels: $*P<0.05$, $**P<0.01$, $***P<0.001$. The figures were obtained from at least four independent experiments with similar patterns.

Results

Effects of NCTD on apoptosis and caspase activation

Accumulating evidence demonstrated that treatment with anticancer drugs has been shown to induce apoptosis, activate

the caspase cascade, and modulate MAPK signaling pathways (Whitmarsh & Davis, 1996; Chen *et al*., 1998). Thus, we first examined the effect of NCTD on apoptosis and caspase activation in the HepG2 human hepatoma cells. As shown in Figure 1a, NCTD induced a time- and dose-dependent apoptotic cell death in HepG2 cells. Exposure of HepG2 cells to 15 μg ml^{-1} NCTD led to proteolytic cleavage of the executioner caspase (caspase-3) and its downstream target PARP (Figure 1b).

Peng *et al*. (2002) reported that activation of caspase-8 is involved in NCTD-triggered apoptosis. Conversely, our previous study showed that caspase-9 and caspase-3 but not caspase-8 were activated in NCTD-treated cells. In this study, we also found that caspase-9 and caspase-3 were activated preceding caspase-8 (Figure 2a). To clarify the role of specific caspases in NCTD-mediated apoptotic process, HepG2 cells were treated with NCTD in the presence or absence of the caspase inhibitor. As depicted in Figure 2b, treatment with caspase-3 and caspase-9 inhibitors drastically blocked the apoptosis induced by NCTD. However, caspase-8 inhibitor just slightly attenuated NCTD-triggered cell death. These results suggest that caspase-9 and caspase-3 cascades play an important role in NCTD-induced apoptotic death in HepG2 cells.

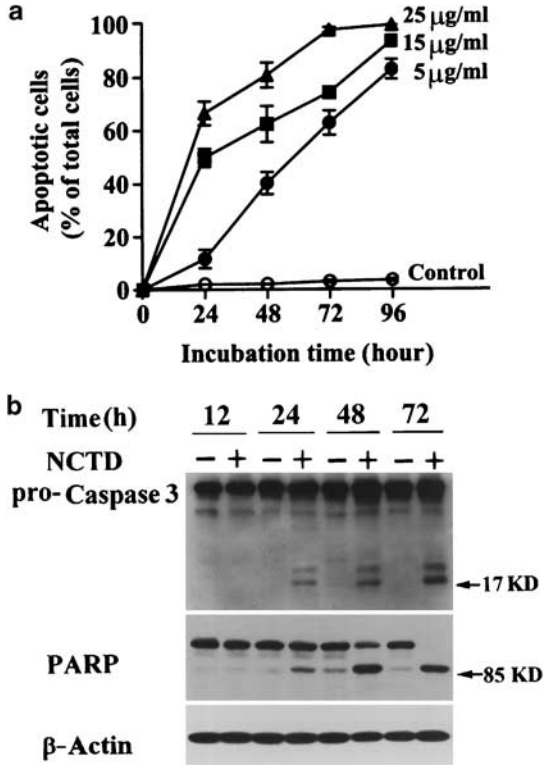

Figure 1 Induction of apoptotic cell death by NCTD. (a) Dose- and time-dependent apoptosis induced by NCTD. HepG2 cells were treated with various concentrations of NCTD (0, 5, 15, and 25 μg ml^{-1}) for 24, 48, 72, and 96 h. After treatment, apoptotic cells were measured by *in situ* TUNEL assay. Data are presented as means ± s.d. of 12 replicates from four separate experiments. (b) NCTD-induced caspase-3 and PARP cleavage. Cells were untreated or treated with 15 μg ml^{-1} NCTD for the indicated time points. Immunoblot analysis was performed using anticaspase-3 and anti-PARP antibodies. β-Actin was used as an internal loading control. The figure was obtained from four separate experiments with similar patterns.

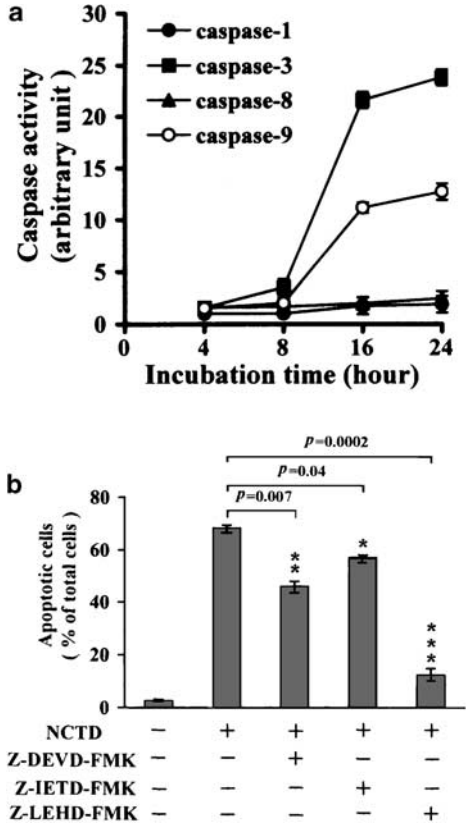

Figure 2 Caspase-9 activation plays a crucial role in NCTD-induced apoptosis. (a) Caspase activation. HepG2 cells were treated with 15 μg ml^{-1} NCTD for 4, 8, 16, and 24 h. After incubation, the cell lysate was prepared, and caspase activity was measured using a fluorogenic substrate. (b) Treatment with caspase inhibitors. Cells were treated with 15 μg ml^{-1} NCTD in the absence or presence of caspase inhibitors for 48 h, and apoptotic cells were estimated by *in situ* TUNEL assay. Data are presented as means ± s.d. of 12 replicates from four separate experiments.

Expression of death-receptor proteins

Western blot analysis was performed to determine the effect of NCTD on the expression levels of death-receptor proteins. As shown in Figure 3, treatment of HepG2 cells with NCTD did not affect the expression levels of all tested death-receptor proteins, including Fas, FasL, DR3, DR4, TNFRI, and TNFRII, suggesting that death-receptor signaling was not required for NCTD-induced apoptosis in HepG2 cells.

Involvement of ERK and JNK in NCTD-induced apoptosis

To characterize the activated status of MAPKs, ERK1/2, JNK, and p38^{MAPK}, immunoblot analysis was performed with phospho-specific antibodies that recognize the active form of each kinase. Exposure to 15 μg ml^{-1} NCTD led to a sustained elevation of the levels of phospho-ERK1/2 and phospho-JNK status, starting at 4 h and continuing through 24 h after treatment (Figure 4a). Immunoblot analysis using antibodies recognizing the total level of each MAPK protein revealed no change in total protein levels (Figure 4a), suggesting that the increases in phospho-specific immunoreactivity were attributable to changes in the phosphorylation status of the existing

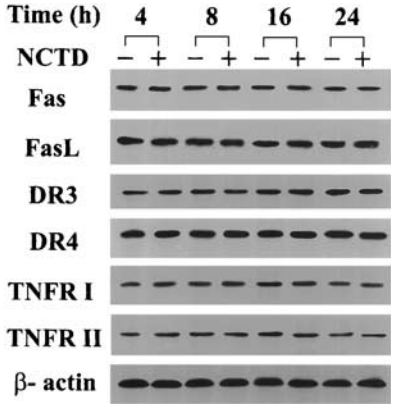

Figure 3 Expression of death-receptor proteins. HepG2 cells were treated with 15 μg ml^{-1} NCTD for 4, 8, 16, and 24 h. After treatment, the cell extracts were prepared. The expression levels of Fas, FasL, TRAIL receptor (DR3 and DR4), and TNF receptor (TNFRI and TNFRII) were detected by immunoblot analysis. β-Actin was used as an internal loading control. The figure was obtained from four separate experiments with similar patterns.

proteins. However, treatment with NCTD neither altered the phosphorylation status nor the total protein content of p38^{MAPK} (Figure 4a). Next, we investigated the possible alterations in ERK, p38^{MAPK}, and JNK activities by using the solid-phase kinase assay. ERK, p38^{MAPK} or JNK protein was immunoprecipitated from whole-cell extracts; *in vitro* MBP (substrate of ERK and p38^{MAPK}) and c-Jun (substrate of JNK) kinase activities were measured. As shown in Figure 4b, there was a persistent increase in ERK and JNK activity in response to NCTD treatment. However, p38^{MAPK} activity was not affected by NCTD. Densitometric analyses showed the ERK activity for the MBP substrate and JNK activity for the GST-c-Jun substrate at 24 h after NCTD treatment to be 4.7$\pm$0.8- and 8.3$\pm$1.2-fold higher than the kinase activity of the controls, respectively. Additional evidence that clarification of NCTD induced the activation of ERK and JNK came from studies in which the enhanced phospho-c-Jun and phospho-c-Myc were observed in NCTD-treated nuclei. Nuclear extracts obtained from HepG2 cells untreated or treated with 15 μg ml^{-1} NCTD for the indicated time points showed that NCTD treatment resulted in increasing the levels of phosphorylated c-Jun and c-Myc, but did not affect the levels of total c-Jun and c-Myc protein contents in NCTD-treated nuclei (Figure 4c). These results indicated that the initiation of apoptosis by NCTD in HepG2 cells was very well correlated with a sustained increase in ERK and JNK activity.

There are contradictory effects of ERK and JNK signaling in cell death or cell survival (Whitmarsh & Davis, 1996; Barr & Bogoyevitch, 2001). To address the role of ERK and JNK activation in NCTD-induced apoptosis, studies were conducted to determine the effects of specific blockade of MAPKs (ERK, JNK or p38MAPK) using selective inhibitors. The activation of ERK requires phosphorylation of MEK1/2 (English *et al*., 1999). U0126 and PD98059 are the selective inhibitors of MEK1/2, bind to the MEK1/2, and prevent its phosphorylation and activation, and subsequently inhibit the phosphorylation and activation of ERK (English *et al*., 1999). Treatment of HepG2 cells with U0126 (20 μM) or PD98059 (40 μM) markedly inhibited the NCTD-induced ERK activity (Figure 5a). *In situ* TUNEL assay showed that treatment with

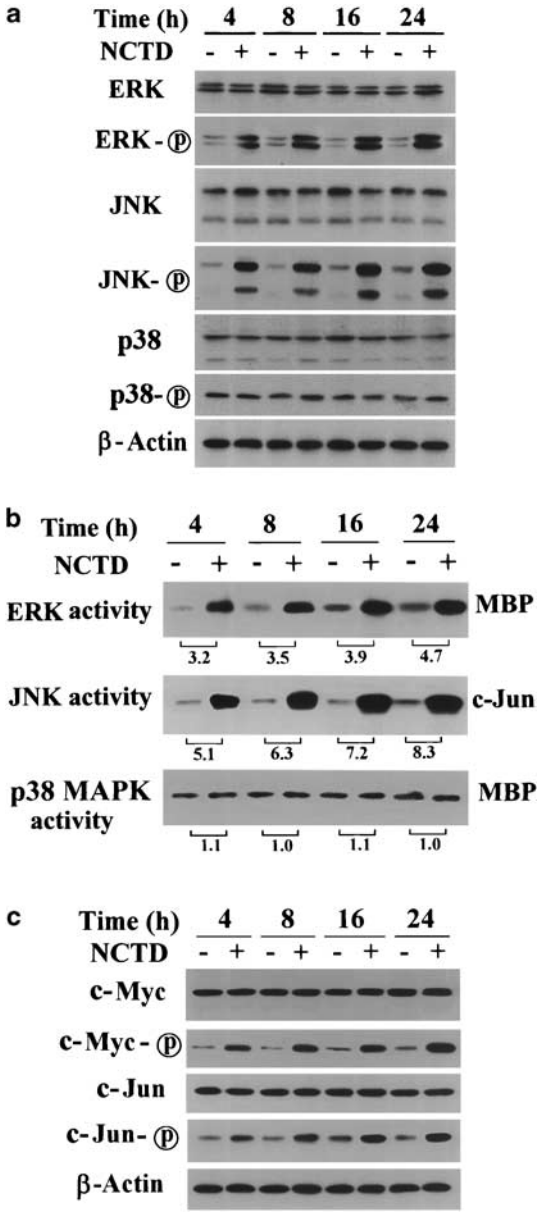

Figure 4 NCTD-induced phosphorylation and activation of MAPK family proteins. (a) Phosphorylation status and expression levels of MAPKs. Treatment of HepG2 cells with 15 μg ml^{-1} NCTD for 4, 8, 16, and 24 h. The expression levels and phosphorylation status were detected by immunoblot analysis. β-Actin was used as an internal loading control. (b) *In vitro* kinase assay. Cells were treated with 15 μg ml^{-1} NCTD for 4, 8, 16, and 24 h. Cell extracts were immunoprecipitated with anti-ERK, anti-p38^{MAPK} or anti-JNK antibody. *In vitro* ERK, p38^{MAPK}, and JNK activity assay was performed using MBP and GST-c-Jun as substrates. Quantitation was by densitometry, and fold activity was calculated relative to that found in vehicle-treated control cultures. The values are presented as means from four independent experiments. (c) Elevation of the levels of phospho-c-Jun and phospho-c-Myc by NCTD. Cells were treated with 15 μg ml^{-1} NCTD for 4, 8, 16, and 24 h. The expression levels and the phosphorylation status of c-Jun and c-Myc were determined by immunoblot analysis. The figures were obtained from four independent experiments with similar patterns.

U0126 or PD98059 alone did not alter the incidence of apoptosis (Figure 5a). However, NCTD-induced apoptotic cell death was significantly attenuated by U0126 and PD98059 (Figure 5a). Moreover, combining NCTD with SP600125, a

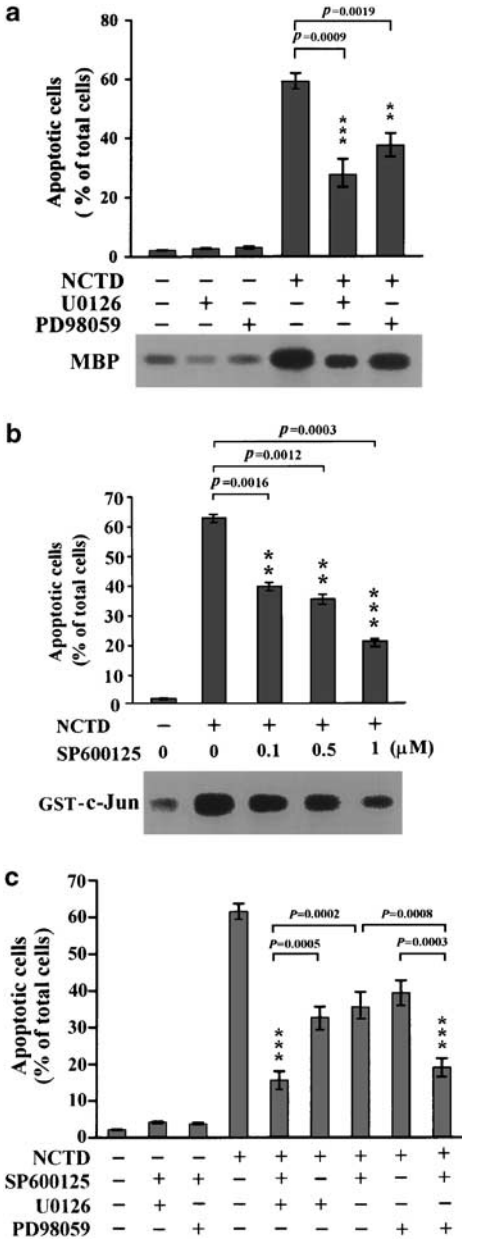

Figure 5 Effects of the ERK and JNK inhibitors on NCTD-induced apoptosis. HepG2 cells were treated without or with (a) ERK inhibitors, U0126 (20 μM) or PD98059 (40 μM), (b) JNK inhibitor, SP600125 (with varying concentrations), or (c) combined treatment with ERK and JNK inhibitors, U0126 (20 μM) + SP600125 (1 μM) or PD98059 (40 μM) + SP600125 (1 μM), in the presence or absence of NCTD (15 μg ml^{-1}) for 48 h. After incubation, the apoptotic cell number was determined by *in situ* TUNEL assay, and the kinase activity was evaluated by the *in vitro* kinase assay using MBP or GST-c-Jun as substrate. Data are presented as means ± s.d. of 12 replicates from four separated experiments. The figures were obtained from four independent experiments with similar patterns.

pharmacological inhibitor of JNK pathway (Weston & Davis, 2002), caused a dose-dependent reduction of NCTD-induced JNK activation, as well as drastically inhibited the cell death induced by NCTD (Figure 5b). However, treatment with a p38^{MAPK} selective inhibitor SB203580 resulted in a significant reduction of p38^{MAPK} activity, but did not affect the apoptosis mediated by NCTD (data not shown). In addition, combined treatment with ERK and JNK inhibitors highly abolished NCTD-induced cell death (Figure 5c). These results suggest that the activation of ERK and JNK pathways, but not p38^{MAPK}, could independently contribute to NCTD-induced apoptosis.

NCTD treatment increased the transactivation potential of AP-1 and NF-κB

It is well documented that ERK and JNK are mediators of signal transduction from the cell surface to the nucleus (Whitmarsh & Davis, 1996; Hidenori, 1999). AP-1 and NF-κB are the nuclear targets of these kinase-signaling pathways (Mercurio & Manning, 1999; Shaulian & Karin, 2002). We further examined whether AP-1 and/or NF-κB could be activated in NCTD-treated HepG2 cells; the DNA-binding activity of AP-1 and NF-κB in nuclear extracts was analyzed by electrophoretic gel mobility shift assay using biotin-labeled oligonucleotides, which contained the consensus-binding motif of AP-1 or NF-κB. As shown in Figure 6, DNA-binding activities of AP-1 and NF-κB were persistently increased after NCTD treatment. This binding was specific since it could be blocked by preincubation with a 100-fold excess amount of unlabeled AP-1 or NF-κB response oligonucleotides. Densitometric analysis showed that the binding activities of AP-1 and NF-κB at 4 h NCTD treatment were increased to 2.9 ± 0.7- and 3.1 ± 0.6-fold vs controls, respectively. The AP-1- and NF-κB-binding activity remained enhanced for 24 h after NCTD treatment, the binding activities of AP-1 and NF-κB were elevated to 8.1 ± 1.0- and 7.7 ± 1.3-fold higher than the binding activity of the controls, respectively.

Suppression of NF-κB or AP-1 signaling attenuated NCTD-induced apoptosis

To define the role of the AP-1 and NF-κB pathways in NCTD-induced apoptosis, we next examined the impact of an AP-1

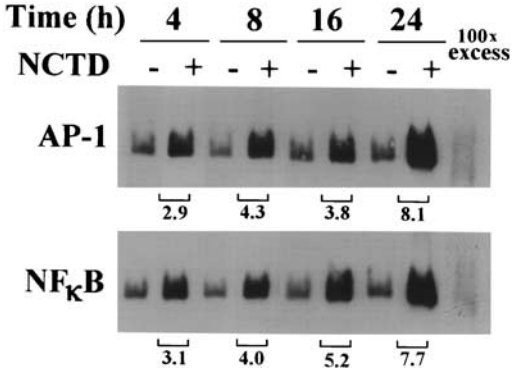

Figure 6 Elevation of AP-1 and NFκB DNA-binding activity by NCTD. HepG2 cells were treatment with 15 μg ml^{-1} NCTD for 4, 8, 16, and 24 h. Nuclear extracts were prepared, and electrophoretic mobility shift assay was performed using biotin-labeled AP-1 or NFκB consensus binding sequence. Specificity of the shift was confirmed by preincubation of 24 h control nuclear extracts with a 100-fold excess of unlabeled AP-1- or NFκB-specific oligonucleotides that almost completely abolished the AP-1 and NFκB shift. Quantitation was by densitometry, and fold activity was calculated relative to that found in vehicle-treated control cultures. The values are presented as means from four independent experiments. The figures were obtained from four independent experiments with similar patterns.

inhibitor, curcumin (Chen & Tan, 1998; Liacini *et al*., 2002), and a NF-κB inhibitor, PDTC (Wright *et al*., 2002). Treatment with curcumin or PDTC alone did not alter the incidence of apoptosis. Combining NCTD with various concentrations of curcumin resulted in a concentration-dependent inhibition of NCTD-induced AP-1 DNA-binding activity and apoptotic cell death (Figure 7a). Moreover, treatment with PDTC also inhibited NCTD-induced NF-κB DNA-binding activity and apoptosis in a dose-dependent manner (Figure 7b). Furthermore, combined treatment with curcumin and PDTC also markedly blocked NCTD-induced cell death (Figure 7c); however, there was no additive effect with the combination of curcumin and PDTC. These results indicated that the AP-1 and NF-κB pathways could provide the apoptotic signal in NCTD-treated HepG2 cells.

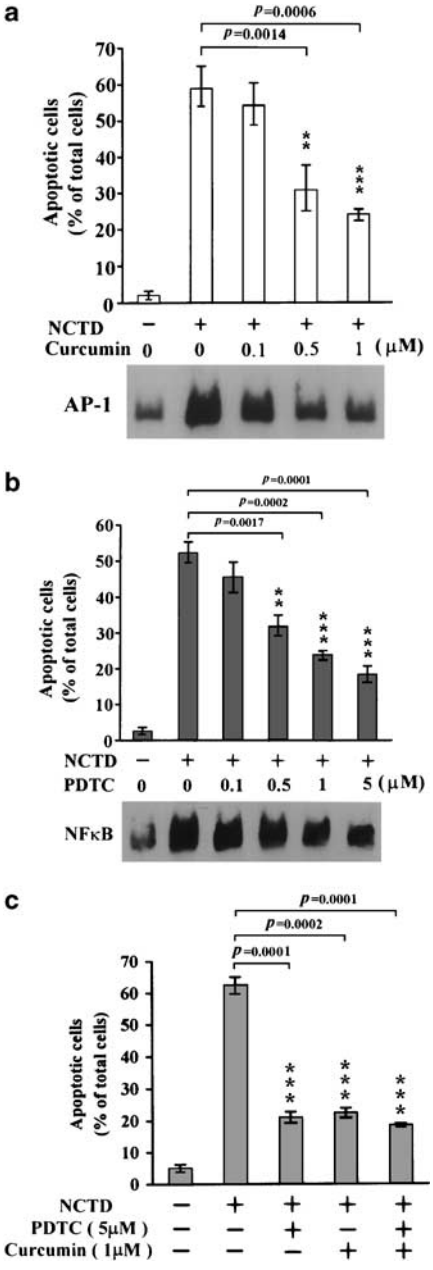

Figure 7 Involvement of AP-1 and NFκB signals in NCTD-induced apoptosis. HepG2 cells were treated with or without 15 $\mu g\,ml^{-1}$ NCTD in the presence or absence of varying concentrations of (a) curcumin, (b) PDTC, or (c) curcumin + PDTC for 48 h. Apoptotic cells were estimated by *in situ* TUNEL assay, and the DNA-binding activity was determined by electrophoretic mobility shift assay. Data are presented as means ± s.d. of 12 replicates from four separated experiments. The figures were obtained from four independent experiments with similar patterns.

Discussion

NCTD is a potent antiproliferative agent *in vitro*, inducing human hepatoma cells to undergo cell cycle arrest and apoptosis (Yang *et al*., 1997; Hong *et al*., 2000; Chen *et al*., 2002; Peng *et al*., 2002). Our previous study determined that NCTD induces a mitochondrial-dependent caspase activation cascade, leading to apoptosis in human hepatoma HepG2 cells (Chen *et al*., 2002). In this study, we found that NCTD-induced apoptosis was accompanied by the strong activation of caspase-9 and caspase-3; however, the activation of caspase-8 was late and slight. Moreover, treatment with the inhibitors of caspase-9 and caspase-3 could markedly prevent the NCTD-mediated apoptosis. In contrast, caspase-8 inhibitor just slightly affected the NCTD-induced apoptosis in HepG2 cells. Data from Western blot analysis indicated that the expression levels of Fas, FasL, TRAIL receptor (DR3 and DR4), and TNF receptors (TNFRI and TNFRII) did not alter upon NCTD treatment. Conversely, upregulation of Fas receptor/ligand and activation of caspase-8 was observed during NCTD-induced apoptosis in human colorectal carcinoma cell lines (Peng *et al*., 2002). Cell-type specific caspase activation reported in retinal ischemia–reperfusion injury indicates that different cell types may use different caspases in the commitment phase of apoptosis, and in response to the same death signal (Katai & Yoshimura, 1999; Singh *et al*., 2001). Thus, the differences between Peng's and our reports may be due to biological differences between the investigated cell types, and the different conditions or analytical methods being used.

The data presented here also demonstrated that ERK and JNK but not $p38^{MAPK}$ were activated in NCTD-treated HepG2 cells. Moreover, selective blockade of these MAPK pathways by pharmacological inhibitors prevented activation of these kinases and inhibited NCTD-induced apoptosis, suggesting that ERK and JNK activation plays an important role in NCTD-induced cell death. It is interesting that both ERK and JNK acted as proapoptotic pathways in our model, as other reports have noted varying influences of MAPKs, particularly ERK, on drug-induced apoptosis (Xia *et al*., 1995; Van den Bringk MRM *et al*., 1999). Accumulating evidence suggests that apoptosis might be induced by the disruption of MAPK signal transduction. Early studies correlated JNK and $p38^{MAPK}$ with apoptosis, whereas ERK was implicated in proliferation and protection from apoptosis (Xia *et al*., 1995). Guyton *et al*. (1996) first reported that ERK activation serves as a survival factor following oxidant injury. Treatment with the pharmacological inhibitor or dominant negative-MEK or –ERK, resulting in reduced ERK activation, was found to sensitize 3T3 cells to hydrogen peroxide. Although the majority of ERK stimuli induce cell proliferation or differentiation, it appears that some stimuli may use the ERK pathway to trigger apoptosis. For example, ligation of the cell surface receptor Fas by its specific ligand or by anti-Fas

antibodies induced an ERK-dependent apoptotic cell death in human neuroblastoma cells (Goillot *et al*., 1997). Furthermore, Yu *et al*. (2001) have reported the involvement of ERK in RRR-a-tocopheryl succinate-induced apoptosis in human breast cancer cells. It should be noted that treatment with RRR-a-tocopheryl succinate induced the activation of ERK beginning 1 h after treatment, and produced a sustained elevation for more than 4 h, and involved a 17-fold increase over control values. These observations imply that the duration and intensity of ERK activation appear to be important in determining cell fate (growth, survival or apoptosis), indicating that ERK may have a dual role in the regulation of cell survival and death. The way in which ERK mediates these opposing cellular processes is still unknown. One possibility is that strong and persistent activation of ERK leads to cell death (Stanciu *et al*., 2000), whereas a transient activation of ERK is associated with proliferation (Fukunaga & Miyamoto, 1998). In support of this, our result showed that NCTD-induced marked ERK activation continued to increase from 4 to 24 h incubation. Strong and prolonged activation of ERK may switch on a downstream signal leading to cell death induced by NCTD.

Growing evidence indicates that activation of JNK kinase pathway plays an important role in apoptosis induced by certain stimuli. For example, in PC12 neuronal cells, activation of the JNK pathway is required for apoptosis induced by nerve growth factor withdrawal (Xia *et al*., 1995). Persistent activation of the JNK has been frequently associated with apoptotic cell death in a variety of cells, including Jurkat T cells, HeLa cells, endothelial cells or rat mesangial cells (Barr & Bogoyevitch, 2001). Inhibition of JNK signaling has been shown to block the apoptosis triggered by ceramide and UV radiation in U937 and Jurkat cells (Chen *et al*., 1996; Verheij *et al*., 1996), the IL-1-induced apoptosis of pancreatic β-cells (Barr & Bogoyevitch, 2001), or the ischemia–reperfusion-induced cell death of rat cardiac myocytes (Barr & Bogoyevitch, 2001). Moreover, activation of the JNK pathway has also been implicated in anticancer drug-induced apoptosis (Herr *et al*., 1997; Chen *et al*., 1999). However, JNK activation has not always been associated with enhanced apoptotic death. For example, JNK activation is not involved in the Fas-induced apoptosis of a proB cell line (Barr & Bogoyevitch, 2001), and is not required for surface IgM-mediated apoptotic death of WEHI 231 B cells (Barr & Bogoyevitch, 2001). Indeed, JNK activation has also been implicated in the cell survival and proliferation (Barr & Bogoyevitch, 2001). In the current study, we showed that NCTD-induced apoptosis was associated with a persistent activation of JNK. Specifically blocking JNK activity by a pharmacological inhibitor SP600125 significantly attenuated NCTD-induced cell death. Our results indicate that JNK activation represents a crucial step in the regulation of apoptosis induced by NCTD. In contrast to ERK and JNK, $p38^{MAPK}$ was not activated by NCTD. Treatment with a $p38^{MAPK}$ selective inhibitor SB203580 did not affect NCTD-induced apoptosis (data not shown), suggesting that $p38^{MAPK}$ pathway was not required for NCTD-induced apoptotic process. What determines whether ERK or JNK will act in a proapoptotic or antiapoptotic fashion remains an important unanswered question, but the kinetics and duration of its activation may be important factors. For example, in situations where ERK or JNK activity enhances survival, activation occurs rapidly, and is more transient (Guyton *et al*., 1996; Ikeyama *et al*., 2001); in situations where it is apoptotic, activation tends to be delayed and sustained (Jimenez *et al*., 1997; Wang *et al*., 2000).

It is well documented that ERK and JNK are mediators of signal transduction from the cell surface to the nucleus. Transcription factors AP-1 and NF-κB are the nuclear targets of these MAP kinase-signaling pathways; activation of ERK and JNK is associated with transactivation of NF-κB and AP-1 factors (Whitmarsh & Davis, 1996; Chen *et al*., 2001). AP-1 has been implicated in many critical cellular processes including apoptosis. The first data regarding the involvement of AP-1 in induction of apoptosis come from studies on the nervous system. Persistent induction of c-Fos in the brains of mice treated with kainic acid induces apoptosis of hippocampal neurons (Smeyne *et al*., 1993). Transient overexpression of c-Jun or c-Fos was found to induce apoptosis in various cell lines (Shaulian & Karin, 2002). Inhibition of c-Jun activity through the expression of a dominant-negative c-Jun mutant can protect neuronal cells from apoptosis induced by nerve growth factor withdrawal (Shaulian & Karin, 2002). It has been reported that JNK-mediated phosphorylation is important for c-Jun-induced apoptosis in neuronal cells. The expression of c-Jun that has been mutated in the JNK phospho-acceptor sites, $c\text{-}Jun^{Ala63/73}$, can block the apoptosis induced by NGF withdrawal (Shaulian & Karin, 2002). Here, we showed that treatment with NCTD caused a JNK-mediated c-Jun phosphorylation and activation. Blockade of AP-1 activity with curcumin, an inhibitor, significantly reduced AP-1 DNA-binding activity, and attenuated NCTD-induced apoptosis, suggesting the involvement of the AP-1-signaling pathway in this NCTD-mediated event.

NF-κB is also known to be involved in the molecular pathway of apoptosis (Chen *et al*., 2001). In general, activation of NF-κB has been reported to protect cells from apoptosis in response to a variety of apoptotic stimuli. Kolenko *et al*. (1999) demonstrated that pharmacological inhibition of NF-κB pathway in PBLs induces caspase-dependent apoptosis. Platelet-activating factor receptor-mediated protection of epidermal cells against TNF-induced apoptosis is *via* an NF-κB activation pathway (Southall *et al*., 2001). The direct evidence for the antiapoptotic effects of NF-κB is provided by gene knockout study in which RelA (p65)-deficient mice die during embryonic development through apoptosis of hepatocytes (Beg *et al*., 1995). However, in a certain case, NF-κB was also considered a proapoptotic factor because of its rapid activation in cells in response to apoptotic signals, and its involvement in the expression of some apoptotic genes, including TNF-α, c-*myc*, and fasL (Chen *et al*., 1999). Du *et al*. (1999) demonstrate that the induction of apoptosis by high glucose was accompanied by NF-κB activation; the apoptotic cell death was prevented by specific p65-NF-κB antisense oligodeoxynucleotides (Du *et al*., 1999). Overexpression of a dominant-negative p65 protein inhibits the apoptosis induced by serum starvation in the established cell line HEK293 (Grimm *et al*., 1996). Mouse p65 knockouts die during embryonic development, and histological examination reveals that this death is probably caused by the massive apoptosis of hepatocytes in these animals (Beg *et al*., 1995). Similarly, our observations showed that NF-κB activation was associated with NCTD-mediated apoptosis; this death event is inhibited by treatment with a specific NF-κB inhibitor PDTC;

we assume that activation of NF-κB appears to be involved in NCTD-induced apoptosis.

In conclusion, NCTD is an anticancer drug that is routinely used to treat hepatoma in China, and can induce apoptotic cell death in several human cancer cell lines. In this study, it is of interest to note that activation of caspase-9, ERK, JNK, AP-1, and N-FκB activation have been demonstrated in human hepatoma HepG2 cells in response to NCTD. Moreover, treatment with the inhibitors of caspase-9, ERK, JNK, AP-1 or NF-κB markedly inhibited the activation of these molecules, and significantly blocked NCTD-induced cell death, suggesting that caspase-9, ERK, JNK, AP-1, and NF-κB are important signals for NCTD-triggered apoptotic process in human hepatoma HepG2 cells. Further study is required to examine the precise upstream signal transduction pathways involved, as well as the downstream targets of AP-1 and NF-κB.

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