Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 67 (2004) 1459–1468
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Cisplatin-induced apoptosis in Hep3B cells: mitochondria-dependent and -independent pathways

Ji Su Kim[a], Jae Myun Lee[a], Yong-Joon Chwae[b], Yeon Hyang Kim[a], Jung Hwan Lee[a], Kunhong Kim[c], Tae Ho Lee[d], Se Jong Kim[a], Jeon Han Park[a,*]

[a]*Department of Microbiology and Brain Korea 21 Project for Medical Sciences, Institute for Immunology and Immunological Diseases, Seoul, South Korea*
[b]*Department of Microbiology, Pochon Cha University College of Medicine, Kyunggi-Do, South Korea*
[c]*Department of Biochemistry and Molecular Biology, Yonsei University College of Medicine, Seoul, South Korea*
[d]*Department of Biology, College of Science, Yonsei University, Seoul, South Korea*

Received 5 August 2003; accepted 4 December 2003

Abstract

Human hepatoma cell lines undergo apoptosis after treatment with cisplatin (CP), by mechanisms that are not fully understood, although our previous study demonstrated that Fas-dependent or -independent pathways are involved. To elucidate the mechanisms of CP-induced apoptosis in Hep3B cells, which are Fas- and p53-negative, we investigated mitochondria associated pathways, the involvement of NF-κB, and p73 activation. Results of Western blot and flow cytometry assay revealed that the translocation of Bax, resulted in the loss of mitochondrial membrane potential ($\Delta\varphi_m$) and the efflux of cytochrome *c* and of second mitochondria-derived activator of caspase/DIABLO from mitochondria into the cytosol. Caspase-3, -8 and -9 were activated by CP treatment, however, CP-induced apoptosis was not completely blocked by pretreating with the pan-caspase inhibitor, benzyloxycarbonyl-valinyl-alaninyl-aspartyl-(*O*-methyl)-fluoromethylketone, indicating that caspase-independent apoptotic pathways might also be involved. RNase protection assay confirmed that NF-κB downregulation leading to the suppression of its target genes, such as *XIAP* and *TRAF2*, and p73 accumulation were also observed in Hep3B cells treated with CP. CP-induced apoptosis was inhibited to some extent by transiently overexpressed p73 dominant negative and XIAP, but not by p73DN or XIAP alone. In conclusion, this study demonstrates that CP-induced apoptosis in Hep3B cells is associated with mitochondrial dysregulation, NF-κB downregulation and p73 accumulation.

Keywords: Cisplatin; Mitochondria; NF-κB; p73; Hep3B

1. Introduction

Cisplatin (CP) is known to induce cell death by generating DNA adducts in many cells [1]. Although CP has been used as a chemotherapeutic agent in many cancers, the molecular mechanism of its anti-cancer activity is not clear. Recently, it was reported that CP induces the mitochondria-mediated apoptotic pathway [2]. The major regulatory steps of apoptosis associated with mitochondria are the disruption of electron transport and of energy metabolism and the opening of a large conductance channel, the permeability transition (PT) pore [3]. The opening of PT pore liberates apoptogenic proteins, such as cytochrome *c* from mitochondria into the cytosol and gives rise to apoptosis [4]. This regulatory step induces the direct activation of caspases by cytochrome *c* [5]. In some cells, the p53-dependent membrane translocation of Bax, which is known as a mitochondrial PT inducer, is triggered by various DNA damaging agents [5]. Transiently overexpressed Bax was found to localize to mitochondria and induce apoptosis [6], and inhibitor of apoptosis protein (IAP) blocked the activations of caspase-3, -6 and -7 by inhibiting the cytochrome *c*-induced activation of caspase-9 [7]. Recently, Smac was identified as a mitochondrial protein that is released with cytochrome *c* from mitochon-

* Corresponding author. Tel.: +82-2-361-5286; fax: +82-2-392-7088.
E-mail address: jhpark5277@yumc.yonsei.ac.kr (J.H. Park).

Abbreviations: CP, cisplatin; PT, permeability transition; Smac, second mitochondria-derived activator of caspase; $\Delta\varphi_m$, mitochondrial membrane potential; PI, propidium iodide; z-VAD-fmk, benzyloxycarbonyl-valinyl-alaninyl-aspartyl-(*O*-methyl)-fluoromethylketone; RPA, RNase protection assay; p73DN, p73 dominant negative; IAP, inhibitor of apoptosis protein.

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2003.12.013

dria into the cytosol by apoptotic stimuli, and which leads to the promotion of caspase activation by binding and neutralizing IAPs [8,9].

NF-κB regulates cell proliferation and inhibits apoptosis by transactivating cell survival factors [10], and NF-κB activation allows some cells to resist chemotherapy [11]. The anti-apoptotic function of NF-κB has been reported to act via the induction of TRAF1 and TRAF2, and the blockade of caspase activation by c-IAP1, c-IAP2 and XIAP [12,13].

p73 has been identified as another member of the p53 family and may function in the regulation of stress response and development [14]. Although the ectopic expression of p73 can transactivate p53-responsive genes and induce cell cycle arrest or apoptosis, in p53-like manner, the role of p73 in apoptosis and tumorigenesis remains unclear [15].

Previously, we reported that CP induces apoptosis in the human hepatoma cell lines, HepG2 and Hep3B, via Fas-dependent and -independent pathway, respectively [16]. Although our previous study showed that the Fas-independent pathway was involved in the apoptosis of CP-treated Hep3B cells, the mechanisms of this process are not fully understood. Therefore, in the present study, to elucidate the mechanism of CP-induced apoptosis in Hep3B cells, we investigated mitochondria associated apoptotic events, NF-κB transcriptional activity, and p73 activation. We suggest that CP-induced apoptosis in Hep3B cells is engaged in mitochondrial dysregulation, NF-κB downregulation, and p73 accumulation.

2. Materials and methods

2.1. Cell culture

The human hepatocellular carcinoma cell line, Hep3B (ATCC HB 8064), was grown in minimum essential medium (GIBCO BRL) supplemented with 10% fetal bovine serum (GIBCO BRL), 2 mM glutamine, 100 unit/mL penicillin and 100 μg/mL streptomycin.

2.2. Reagents and plasmids

CP was purchased from Sigma Chemical Co. Rabbit anti-Smac antibody was kindly provided from Dr. X. Wang (Howard Hughes Medical Institute and Department of Biochemistry, University of Texas Southwestern Medical Center at Dallas). Mouse anti-HSP60 and rabbit anti-p65 antibodies were purchased from Santa Cruz Bio Technology. Mouse anti-cytochrome *c*, rabbit anti-Bax, and anti-caspase-3, -8, and -9 antibodies were purchased from PharMingen. Mouse anti-α tubulin antibody was purchased from Oncogene Research Products. Mouse anti-p73 antibody was purchased from NeoMarkers. FITC-conjugated mouse anti-CD8 antibody was purchased from Becton-Dickinson. The caspase inhibitor, benzyloxycarbonyl-valinyl-alaninyl-aspartyl-(*O*-methyl)-fluoromethylketone (z-VAD-fmk) (R&D System) was added to the culture to a final concentration of 100–300 μM and incubated for 1 hr before CP treatment. p73-α and -β constructs were kindly donated by Dr. J.Y.J. Wang (Department of Biology and the Cancer Center, University of California). p73DN was a gift from Dr. W.G. Kaelin Jr. (Dana-Farber Cancer Institute and Harvard Medical School).

2.3. Annexin V and propidium iodide (PI) staining

Annexin V and PI staining was performed using an Annexin V–FITC Apoptosis Kit (BioSource) to measure apoptosis. Briefly, after treatment with 15 μg/mL CP, cells were washed with PBS twice, collected and resuspended in 100 μL of 1× Annexin V–FITC binding buffer. Five microliters of Annexin V–FITC conjugate and 10 μL of PI buffer were added, and the cells were then incubated at room temperature for 15 min in the dark. After adding 400 μL of 1× Annexin V–FITC binding buffer, the cells were analyzed using a FACScan flow cytometer (Becton Dickinson).

2.4. NF-κB reporter assay

Cells (2×10^6 cells per well) were seeded onto a 6-well plate, and 0.75 μg p2x NF-κB-Luc and 0.25 μg pCMV-β-gal plasmids were cotransfected into the cells using Lipofectamine (GIBCO BRL). After 24 hr, the cells were treated with CP and harvested at the indicated times. Luciferase activity was determined using a Luciferase Assay System (Promega), and normalized against β-galactosidase activity [17].

2.5. RNase protection assay (RPA)

RPA was performed by using a RiboQuant™ multi-probe RNase protection assay system (PharMingen) following the standard protocol provided by the manufacturer. Briefly, anti-sense probes for hAPO-5 were synthesized using an *in vitro* transcription kit (PharMingen) in the presence of 137.5 μM rNTPs, and 100 μCi [^{32}P]UTP (3000 Ci/mmol; NEN). Total RNA was prepared using an RNeasy kit (Qiagen Inc.) and 10 μg RNA was hybridized with ^{32}P-labeled anti-sense probes. After hybridization, 20 ng RNase A and 50 U RNase T1 were added to digest the unhybridized RNA. The duplex RNA hybrids were then resolved on 5% denaturing polyacrylamide gels containing 8 M urea and analyzed by autoradiography.

2.6. Determination of $\Delta\varphi_m$

To detect $\Delta\varphi_m$ changes, cells (2×10^5 cells per well) treated with CP were harvested at the indicated times and stained with 10 μg/mL JC-1 (Molecular Probes) for 10 min

at room temperature in the dark. Cells were then washed twice with cold PBS, and resuspended in 400 μL PBS for flow cytometry.

2.7. Separation of the cytosolic and mitochondrial proteins

Cytosolic and mitochondrial proteins were separated as described by Gao *et al.* [5] with minor modifications. Briefly, cells treated with CP were collected and suspended in mitochondria isolation buffer (20 mM Hepes–KOH, pH 7.5, 210 mM sucrose, 70 mM mannitol, 1 mM EDTA, 1 mM DTT, 1.5 mM $MgCl_2$, 10 mM KCl) and protease inhibitor cocktail (Boehringer Mannheim) supplemented with 10 μM digitonin (Sigma). Suspensions were incubated at 37° for 10 min and centrifuged at 12,000 *g* for 15 min. The supernatant (cytosolic fraction) and pellet containing the mitochondria were collected for Western blotting.

2.8. Western blot

After CP treatment, cells were lysed in RIPA buffer (50 mM Tris–HCl, pH 7.4, 0.5 mM EDTA, 1.8 μg/mL aprotinin, 100 mM NaCl, 0.2% NP-40, 2 mM $MgCl_2$, 0.5 mM PMSF), and the lysates were cleared by centrifugation at 12,000 *g* for 15 min. One hundred micrograms of cell lysates were used for 8–15% SDS–PAGE, which was followed by Western blotting using the appropriate antibodies. Proteins were visualized by using Western blotting Luminol Reagent (Santa Cruz Bio Technology).

2.9. Transfection

Cells (2×10^5 cells per well) seeded onto a 12-well plate, were co-transfected with 0.75 μg p73-α or -β construct and 0.25 μg pCDNA-CD8TN plasmid expressing an extracellular and a transmembrane domain of human CD8 α chain [18] using Lipofectamine (Invitrogen). After 48 and 72 hr of transfection, cells were stained with FITC-conjugated mouse anti-CD8 antibody and cell death was analyzed by FACS. To determine the effect of p73DN or XIAP on CP-induced apoptosis in Hep3B cells, cells (2×10^6 cells per well) seeded onto a 6-well plate, were transfected with 10 μg p73DN and/or XIAP constructs using Lipofectamine (Invitrogen). After 48 hr of transfection, cells were treated with CP for 48 hr and stained with Annexin V–FITC conjugate and PI.

3. Results

3.1. Translocation of Bax to mitochondria in Hep3B cells treated with CP

Apoptosis was found to occur in Hep3B cells treated with CP by time-dependent manner (data not shown),

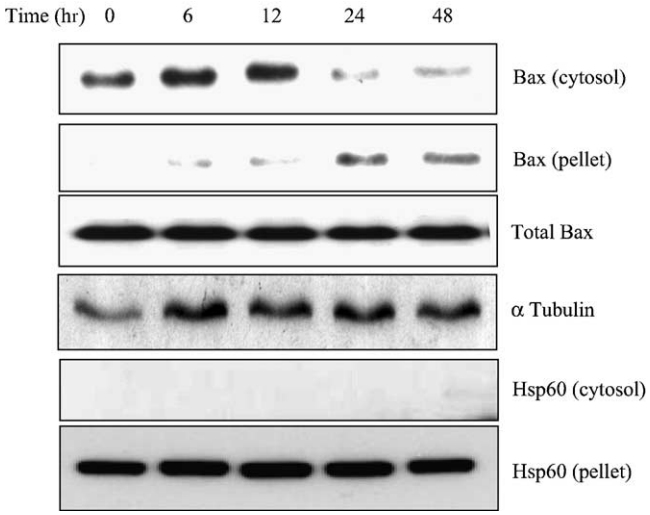

Fig. 1. Translocation of Bax to mitochondria by CP. To detect Bax translocation from the cytosol to mitochondria, samples were fractionated from Hep3B cells treated with 15 μg/mL CP, and blotted with anti-Bax antibody. To determine the total level of Bax, total cell extracts from Hep3B cells were subjected to Western blot. The membrane was stripped and reprobed with anti-α tubulin antibody as a loading control. To confirm fractionation, each fraction was blotted with anti-HSP60 antibody.

which is consistent with our previous study [16]. Recently, it was reported that CP-induced apoptosis involves Bax translocation to mitochondria in mouse collecting duct cells [1]. To investigate whether CP induces Bax translocation in Hep3B cells undergoing apoptosis, we examined the intracellular localization of Bax. Fractionated cytosolic sample and pellet containing mitochondria of Hep3B cells undergoing apoptosis were subjected to Western blotting. Cytosolic Bax was found to have decreased and mitochondrial Bax accumulated in a time-dependent manner (Fig. 1), indicating that CP induces the translocation of Bax from the cytosol to mitochondria during the apoptosis of Hep3B cells.

3.2. Reduction of $\Delta\varphi_m$ in Hep3B cells treated with CP

Since the loss of $\Delta\varphi_m$ is one of the early apoptotic changes, which is induced by Bax through an interaction with the PT pores [19,20], we investigated whether $\Delta\varphi_m$ is reduced in Hep3B cells showing CP-induced Bax translocation. $\Delta\varphi_m$ was measured using the lipophilic cation dye, JC-1, which produces a color change from red to green as the mitochondrial membrane becomes depolarized [20]. Figure 2 shows that cells with depolarized mitochondria gradually increased according to CP treatment time.

3.3. Release of cytochrome c and Smac from mitochondria into the cytosol by CP

Since it has been reported that cytochrome *c* is released from mitochondria into the cytosol during Bax translocation-mediated apoptosis and anticancer drugs-induced apoptosis [6,20], we expected that cytochrome *c* might

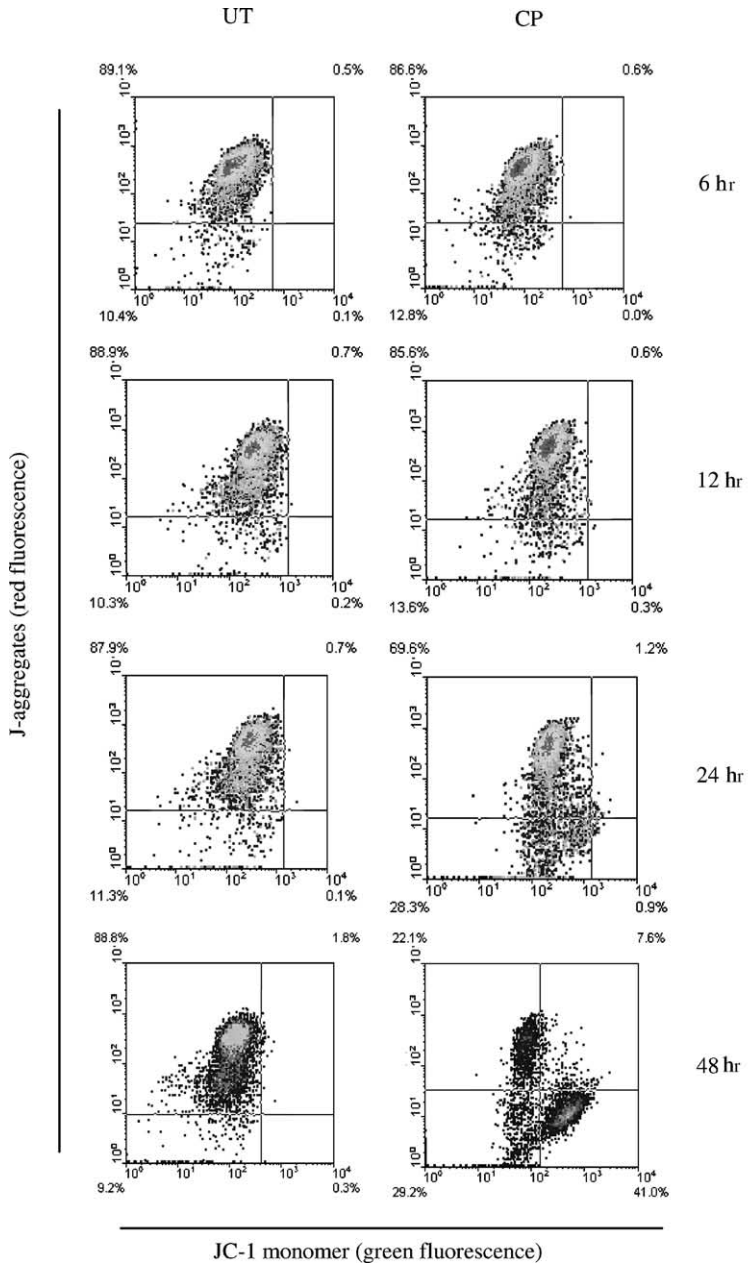

Fig. 2. The loss of $\Delta\varphi_m$ by CP. For the cytofluorometric analysis of $\Delta\varphi_m$, Hep3B cells treated with (CP) or without (UT) 15 μg/mL CP were stained with JC-1. Green emission was measured in fluorescence channel 1 (*X*-axis) and red emission in channel 2 (*Y*-axis). The data shown is representative of two independent experiments.

be released from mitochondria into the cytosol in accord with the mitochondrial translocation of Bax and the reduction of $\Delta\varphi_m$. To observe the efflux of cytochrome *c* from mitochondria, cytosolic and pellet fractions isolated at the various times were subjected to Western blotting. It was found that CP induced the release of cytochrome *c* from mitochondria into the cytosol in a time-dependent manner (Fig. 3A).

Recent studies associated with cytochrome *c* have shown that another mitochondrial protein, Smac, is released along with cytochrome *c* during apoptosis, and that this then neutralizes IAPs [8,9]. To investigate the release of Smac in Hep3B cells undergoing CP-induced apoptosis, we determined cytosolic and mitochondrial Smac by immunoblotting fractionated cell lysates. It appears that Smac is released with cytochrome *c* from mitochondria into the cytosol during CP-induced apoptosis in Hep3B cells in a time-dependent manner (Fig. 3B).

3.4. Partial inhibition of CP-induced apoptosis by a pan-caspase inhibitor

Cytochrome *c* and Smac released into cytosol are known to promote caspase activation [21]. To confirm caspase activation, Western blotting was performed for caspase-3, -8, and -9. CP led to the cleavage of procaspase-3, -8 and -9,

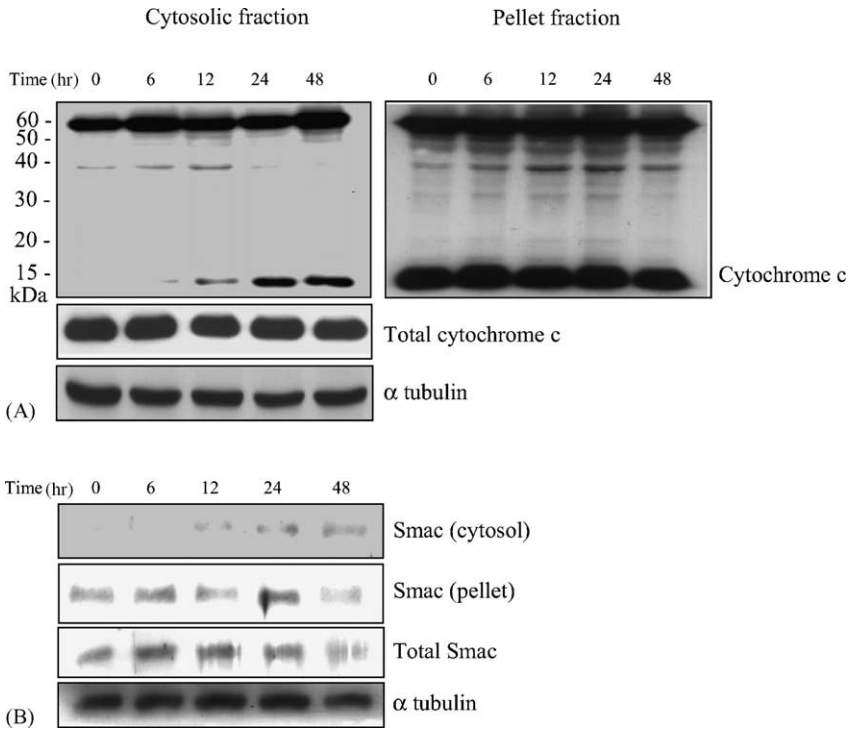

Fig. 3. Release of cytochrome *c* and Smac from mitochondria into the cytosol by CP. CP-treated Hep3B cells were harvested at various time intervals and cell lysates were fractionated into cytosolic and pellet fractions. (A) Immunoblot was performed upon each fraction with anti-cytochrome *c* antibody. (B) Cytosolic and mitochondrial Smac were immunoblotted in cytosolic and pellet fractions using anti-Smac antibody. Total extracts were analyzed to examine the effect of CP on the basal expression level of cytochrome *c* and Smac. The membrane was stripped and reprobed with anti-α tubulin antibody as a loading control.

and their cleaved forms were detected until 48 hr (caspase-3, -8) and 12 hr (caspase-9), respectively (Fig. 4A). To examine whether the pan-caspase inhibitor, z-VAD-fmk, inhibits caspase-dependent apoptosis by CP, cells were pretreated with various concentration of z-VAD-fmk for 1 hr before adding CP, and cytotoxicity was measured by Annexin V and PI staining. CP-induced apoptosis was found to be decreased in Hep3B cells by z-VAD-fmk in a dose-dependent manner (Fig. 4B). However, this apoptosis was not completely inhibited by z-VAD-fmk, we speculated that other apoptotic (mitochondria-independent) pathways might be involved, and thus, we searched for NF-κB and p73-related events.

3.5. Downregulation of NF-κB transcriptional activity by CP

To investigate the effect of CP on NF-κB in Hep3B cells, we examined the transcriptional activity and expression level of NF-κB. We determined the basal expression of p65, because we found that the NF-κB in Hep3B cells is mainly composed of p50 and p65, which is the prototype of NF-κB heterodimers [22], by supershift assay with anti-p50 and anti-65 antibodies (data not shown). Consistent with Mandonado's report [23], the expression level of p65 was unchanged by CP treatment, however, CP steadily downregulated NF-κB transcriptional activity in Hep3B cells in a time-dependent manner (Fig. 5A). In terms of the anti-apoptotic role of NF-κB, it has been reported that its target genes contain cell survival related genes, such as *c-IAP1*, *c-IAP2*, *TRAF1*, *TRAF2* [12], *XIAP*, *BCL-2* and BCL_{XL} [13]. To determine the effect of CP on the transcription of NF-κB target genes, RPA was performed with APO-5. We found that *XIAP* and *TRAF2* mRNA transcript levels were dramatically reduced in Hep3B cells treated with CP, in a time-dependent manner (Fig. 5B). These results imply that the CP-induced downregulation of *XIAP* and *TRAF2* transcription might play a role in enhancing the apoptotic signal by affecting cell survival.

3.6. Effect of p73 and XIAP on Hep3B cells apoptosis induced by CP

It has been reported that p73 is accumulated and is activated by CP in HCT116 containing wild type p53, and that its proapoptotic function is enhanced by c-Abl under the presence of the intact mismatch repair gene, *MLH1* [24]. To verify that p73 plays a role in apoptosis in p53-negative Hep3B cells treated with CP, we checked endogenous MLH1 and c-Abl expressions. Both MLH1 and c-Abl were detected in Hep3B cells (data not shown), though c-Abl was time dependently reduced by CP, which is consistent with Gong's report [24]. Since MLH1 and c-Abl were constitutively expressed in Hep3B cells, we

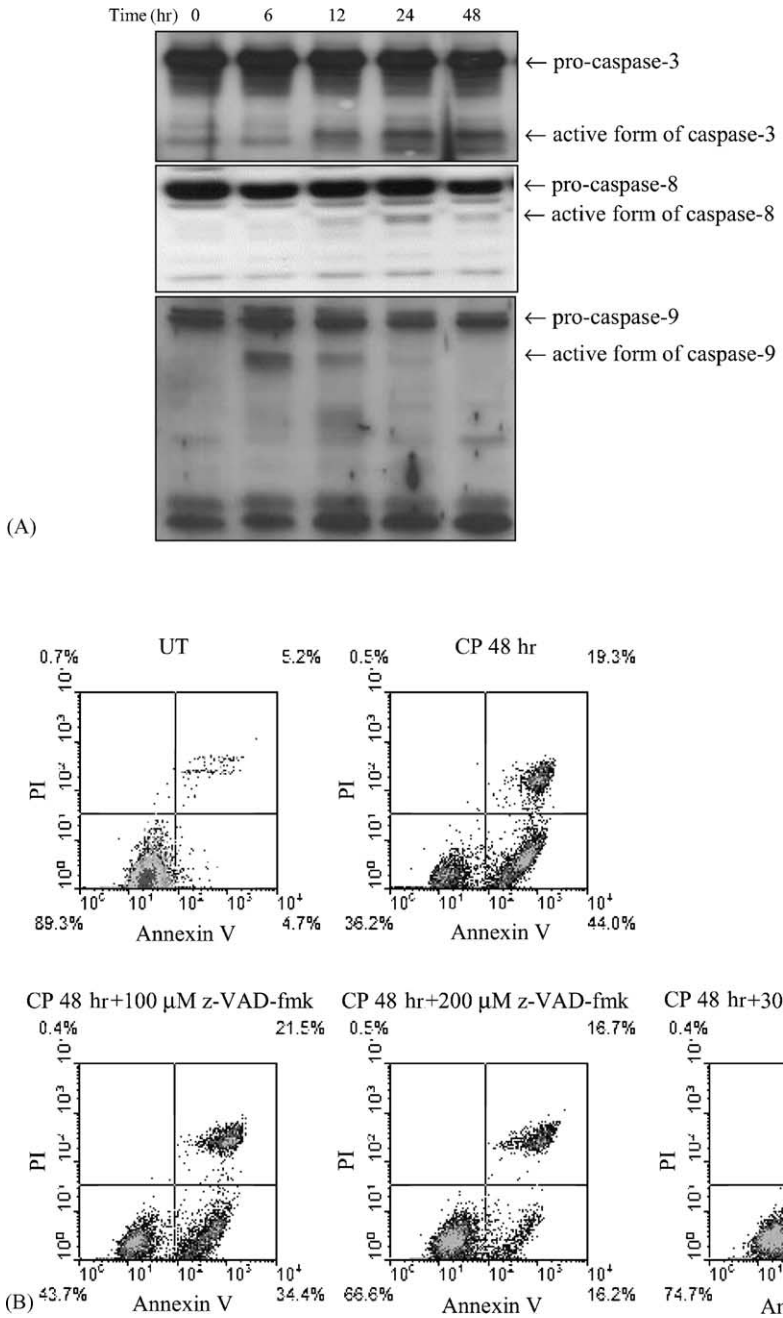

Fig. 4. Activation of caspases during CP-induced apoptosis. (A) Total cell lysates from Hep3B cells treated with 15 μg/mL CP at the various times were immunoblotted with anti-caspase-3, -8 and -9 antibodies, respectively. (B) To inhibit the action of caspase, 100–300 μM z-VAD-fmk was pretreated 1 hr before addition of CP. Cells were then harvested at 48 hr and stained with Annexin V–FITC and PI, and analyzed by flow cytometry. Data is representative of three independent experiments.

examined the expression of p73 in Hep3B cells treated with CP. An accumulation of p73 was detected in a time-dependent manner (Fig. 6A). Thus, we speculated that accumulated p73 might play a role in CP-induced mitochondria-independent apoptosis in Hep3B cells together with NF-κB downregulation.

To examine the role of p73 in the apoptosis of Hep3B cells, p73-α or -β construct was co-transfected with pCDNA-CD8TN, and its effect on apoptosis was evaluated by the percentage of CD8+ cell population. CD8+ cell population was decreased by p73-α or -β in a time-dependent manner, indicating that the expression of p73 in Hep3B cells lead to apoptotic cell death (Fig. 6B). To confirm the role of p73 in CP-induced apoptosis, we transiently transfected p73DN and/or XIAP constructs into Hep3B cells, and then treated the cells with CP, apoptosis was determined by Annexin V and PI staining. Ectopically expressed p73DN or XIAP alone did not block CP-induced apoptosis, however, the coexpression of p73DN and XIAP did inhibit CP-induced apoptosis to some extent (Fig. 6C),

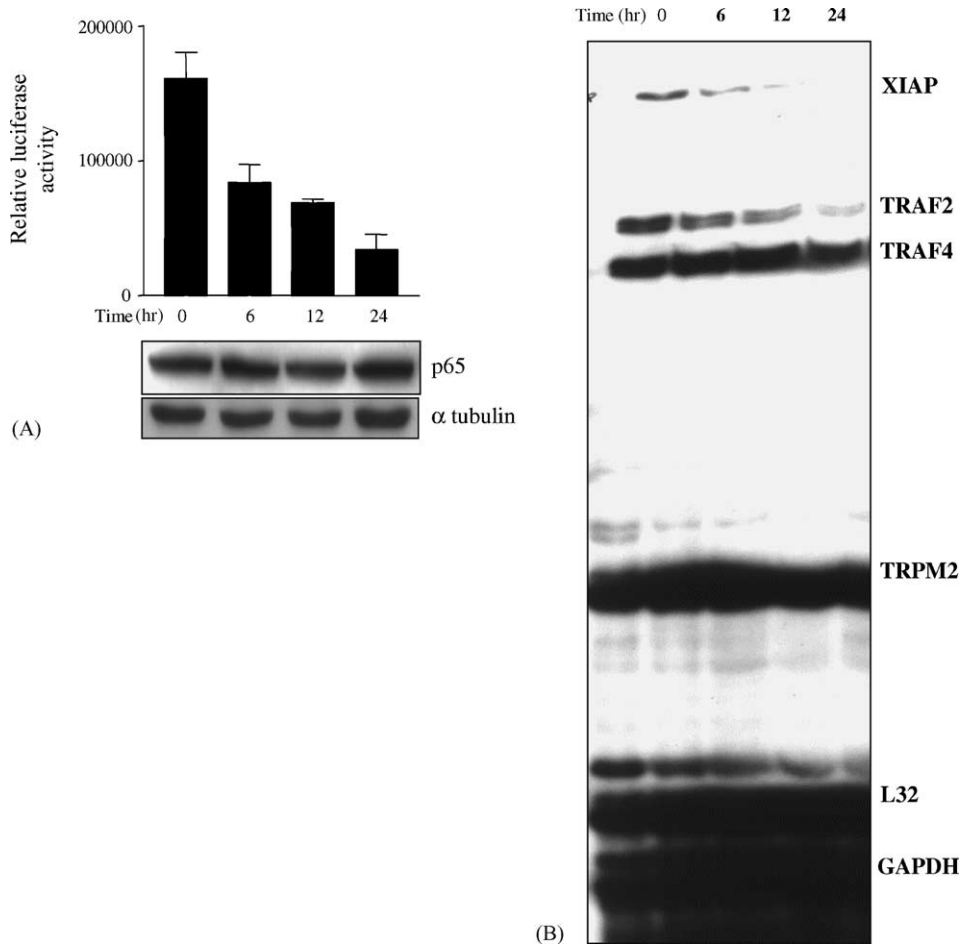

Fig. 5. Downregulation of NF-κB transcriptional activity by CP. (A) Cells were transiently transfected with p2x NF-κB-Luc and CMV-β gal constructs. Twenty-four hours post-transfection, the cells were treated with 15 μg/mL CP and harvested at the indicated times. NF-κB transcriptional activity was analyzed by luciferase assay and normalized vs. β-galactosidase activity. The data shown is the means and standard deviations of experiments performed in triplicate. Total cell extracts from Hep3B cells treated with 15 μg/mL CP, were Western blotted with anti-p65 antibody. The membrane was then stripped and reprobed with anti-α tubulin antibody as a loading control. (B) Total RNA extracted from Hep3B cells treated with 15 μg/mL CP was isolated and analyzed using a hAPO-5 template set by RPA at the indicated times. House keeping genes, *L32* and *GAPDH*, were used as internal controls.

indicating that p73DN and XIAP are synergistically involved in the inhibition of CP-induced Hep3B apoptosis.

4. Discussion

Previously we demonstrated that CP-induced apoptosis in the hepatoma cell lines, HepG2 and Hep3B, occurs via Fas-dependent and -independent pathway, respectively [16]. In the present study, to elucidate the possible mechanism of the Fas-independent pathway induced by CP in Fas- and p53-negative Hep3B cells, we investigated mitochondrial involvement, NF-κB activity and p73 function. We found that CP-induced apoptosis in Hep3B cells is associated with mitochondrial dysregulation, NF-κB downregulation, and p73 accumulation.

CP has been shown to induce apoptosis by releasing cytochrome *c* and activating caspase-3 in breast cancer cells [25] and by triggering Bax translocation in mouse collecting duct cells [1]. In terms of mitochondrial dysregulation, we detected the redistribution of Bax to mitochondria and the loss of $\Delta\varphi_m$, which resulted in the efflux of cytochrome *c* and Smac, and thereby the activation of caspases. However, this apoptosis was not completely blocked by various concentrations of z-VAD-fmk and this result is consistent with Gross' report which showed that apoptosis caused by Bax translocation and mitochondrial dysfunction was not blocked with caspase inhibitor [26]. Our results strongly imply that (a mitochondrial dysregulation-induced) caspase activation-independent apoptotic pathways might also be involved in CP-induced apoptosis in Hep3B cells.

Recently, it was reported that some anti-cancer chemotherapeutic drugs induce the suppression of NF-κB activation, and that this results in the sensitization of cancer cells to apoptosis in cell type specific and signal-dependent manner [27]. This occurs because NF-κB is crucial to cell survival as it regulates the transcription of anti-apoptotic genes, such as *XIAP*, which blocks caspases and neutralizes by interacting with cytosolic Smac [28]. Thus, the downregulation of NF-κB and the reduction of XIAP

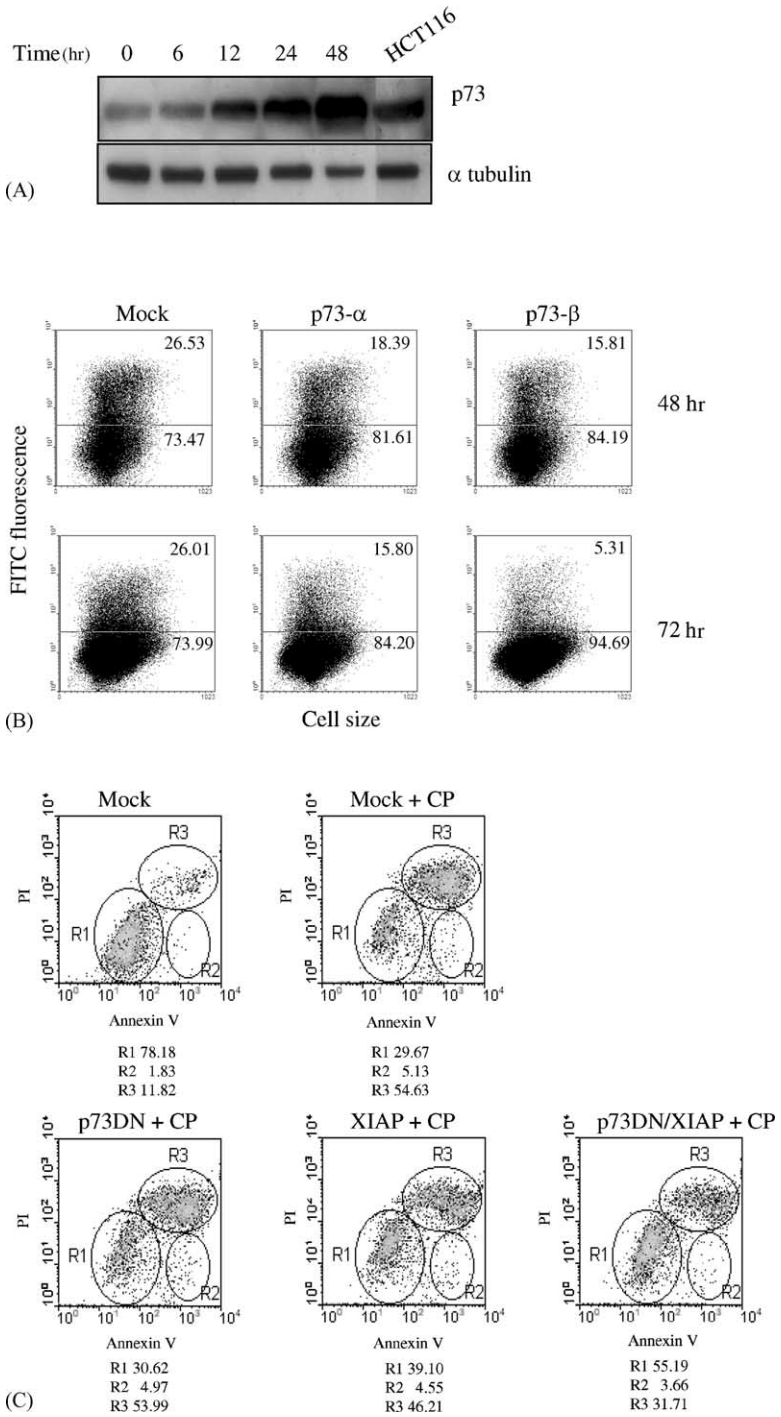

Fig. 6. The roles of p73 and XIAP in CP-induced apoptosis. (A) Total cell extracts from Hep3B cells treated with 15 μg/mL CP were immunoblotted with anti-p73 antibody. The membrane was stripped and reprobed with anti-α tubulin antibody as a loading control. HCT116 cells; a positive control of p73. (B) Hep3B cells were cotransfected with 0.25 μg pCDNA-CD8TN and 0.75 μg p73-α or -β construct, then stained with FITC-conjugated anti-CD8 antibody and analyzed by FACS. The horizontal line represents an arbitrary division between CD8-positive cells (top) and -negative cells (bottom). The data shown is representative of three independent experiments. (C) To examine the effect of p73DN and XIAP on the inhibition of CP-induced apoptosis, p73DN and/or XIAP constructs were transiently transfected in Hep3B cells. Twelve hours post-transfection, 15 μg/mL CP was added for 48 hr, and the cells were Annexin V–FITC and PI stained, and assessed by flow cytometry. The data shown is representative of three independent experiments.

transcripts in Hep3B cells treated by CP might reduce their chances of survival (independently of mitochondrial dysregulation), and the release of Smac into the cytosol might affect both caspase activation and the enhancement of apoptosis.

It has been reported that the accumulation and ectopic expression of p73 induce apoptosis [24,29], and it is known that Hep3B cells have nonfunctional p53 [30]. Therefore, we speculated that p73 accumulation by CP might play a role in mitochondria-independent apoptosis. Transiently

overexpressed p73-induced apoptosis in Hep3B cells, but p73DN or XIAP alone did not block CP-induced apoptosis, confirming Ferreira's finding that CP-induced apoptosis was not blocked by XIAP alone [31]. However, the coexpression of p73DN and XIAP inhibited CP-induced apoptosis in Hep3B cells to some extent. These data support the notion that a CP-induced apoptotic mechanism in Hep3B cells might also be controlled by a p73-associated pathway induced by DNA damage and by the suppression of XIAP by downregulated NF-κB activity. However, because of caspase-8 activation, we cannot exclude the possibility of death receptor-mediated caspase activation in Fas-negative Hep3B cells [16]. To investigate this possibility, we examined the transcripts of some death receptors in Hep3B cells treated with CP. CP did not show any significant effect on the level of transcripts of death receptor genes such as, *Fas*, *DR3*, *DR4*, and *DR5* (our unpublished observations). Downregulation of *BCL-2* by p53 has also been reported in HeLa cells treated with CP [23]. However, our RPA result shows that CP did not display any significant effect on the transcription of the *BCL-2* genes family in Hep3B cells (our unpublished observations), which might be explained by the lack of functional p53 in Hep3B cells.

Although we could not completely rule out the involvement of death receptor-mediated apoptosis, in this study, CP-induced apoptosis was found to be promoted by the translocation of Bax to mitochondria, which led to mitochondrial dysregulation involving cytochrome *c* and Smac release and caspase activation, and by an accumulation of p73. Downregulation of NF-κB, which decreases the transcriptions of *XIAP*, and *TRAF-2* was also involved in CP-induced apoptosis of Hep3B cells.

Acknowledgments

We would like to thank Dr. Jean Y.J. Wang for p73-α and -β constructs, Dr. William G. Kaelin Jr. for p73 DN construct, and Dr. Xiaodong Wang for anti-Smac antibody. This research was supported by a grant from the Korea Science and Engineering Foundation (2000-2-20900-011-3).

References

[1] Lee RH, Song JM, Park MY, Kang SK, Kim YK, Jung JS. Cisplatin-induced apoptosis by translocation of endogenous Bax in mouse collecting duct cells. Biochem Pharmacol 2001;62:1013–23.

[2] Park MS, De Leon M, Devarajan P. Cisplatin induces apoptosis in LLC-PK1 cells via activation of mitochondrial pathways. J Am Soc Nephrol 2002;13:858–65.

[3] Jiang S, Cai J, Wallace DC, Jones DP. Cytochrome *c*-mediated apoptosis in cells lacking mitochondrial DNA. Signaling pathway involving release and caspase 3 activation is conserved. J Biol Chem 1999;274:29905–11.

[4] Bortner CD, Cidlowski JA. Cellular mechanisms for the repression of apoptosis. Annu Rev Pharmacol Toxicol 2002;42:259–81.

[5] Gao CF, Ren S, Zhang L, Nakajima T, Ichinose S, Hara T, Koike K, Tsuchida N. Caspase-dependent cytosolic release of cytochrome *c* and membrane translocation of Bax in p53-induced apoptosis. Exp Cell Res 2001;265:145–51.

[6] Rosse T, Olivier R, Monney L, Rager M, Conus S, Fellay I, Jansen B, Borner C. Bcl-2 prolongs cell survival after Bax-induced release of cytochrome *c*. Nature 1998;391:496–9.

[7] Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM, Alnemri ES, Salvesen GS, Reed JC. IAPs block apoptotic events induced by caspase-8 and cytochrome *c* by direct inhibition of distinct caspases. EMBO J 1998;17:2215–23.

[8] Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome *c*-dependent caspase activation by eliminating IAP inhibition. Cell 2000;102:33–42.

[9] Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell 2000;102:43–53.

[10] Plumpe J, Malek NP, Bock CT, Rakemann T, Manns MP, Trautwein C. NF-kappaB determines between apoptosis and proliferation in hepatocytes during liver regeneration. Am J Physiol Gastrointest Liver Physiol 2000;278:G173–83.

[11] Wang CY, Cusack Jr JC, Liu R, Baldwin Jr AS. Control of inducible chemoresistance: enhanced anti-tumor therapy through increased apoptosis by inhibition of NF-kappaB. Nat Med 1999;5:412–7.

[12] Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin Jr AS. NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science 1998; 281:1680–3.

[13] Tang G, Minemoto Y, Dibling B, Purcell NH, Li Z, Karin M, Lin A. Inhibition of JNK activation through NF-kappaB target genes. Nature 2001;414:313–7.

[14] Levrero M, De Laurenzi V, Costanzo A, Gong J, Wang JY, Melino G. The p53/p63/p73 family of transcription factors: overlapping and distinct functions. J Cell Sci 2000;113(Pt 10):1661–70.

[15] Zhu J, Jiang J, Zhou W, Chen X. The potential tumor suppressor p73 differentially regulates cellular p53 target genes. Cancer Res 1998;58:5061–5.

[16] Jiang S, Song MJ, Shin EC, Lee MO, Kim SJ, Park JH. Apoptosis in human hepatoma cell lines by chemotherapeutic drugs via Fas-dependent and Fas-independent pathways. Hepatology 1999;29: 101–10.

[17] Hsu H, Xiong J, Goeddel DV. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell 1995;81:495–504.

[18] Chwae YJ, Chang MJ, Park SM, Yoon H, Park HJ, Kim SJ, Kim J. Molecular mechanism of the activation-induced cell death inhibition mediated by a p70 inhibitory killer cell Ig-like receptor in Jurkat T cells. J Immunol 2002;169:3726–35.

[19] Narita M, Shimizu S, Ito T, Chittenden T, Lutz RJ, Matsuda H, Tsujimoto Y. Bax interacts with the permeability transition pore to induce permeability transition and cytochrome *c* release in isolated mitochondria. Proc Natl Acad Sci USA 1998;95:14681–6.

[20] Sanchez-Alcazar JA, Khodjakov A, Schneider E. Anticancer drugs induce increased mitochondrial cytochrome *c* expression that precedes cell death. Cancer Res 2001;61:1038–44.

[21] Adrain C, Creagh EM, Martin SJ. Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. EMBO J 2001;20:6627–36.

[22] Li X, Stark GR. NFkappaB-dependent signaling pathways. Exp Hematol 2002;30:285–96.

[23] Maldonado V, Melendez-Zajgla J, Ortega A. Modulation of NF-kappa B, and Bcl-2 in apoptosis induced by cisplatin in HeLa cells. Mutat Res 1997;381:67–75.

[24] Gong JG, Costanzo A, Yang HQ, Melino G, Kaelin Jr WG, Levrero M, Wang JY. The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage. Nature 1999;399:806–9.
[25] Blanc C, Deveraux QL, Krajewski S, Janicke RU, Porter AG, Reed JC, Jaggi R, Marti A. Caspase-3 is essential for procaspase-9 processing and cisplatin-induced apoptosis of MCF-7 breast cancer cells. Cancer Res 2000;60:4386–90.
[26] Gross A, Jockel J, Wei MC, Korsmeyer SJ. Enforced dimerization of BAX results in its translocation. EMBO J 1998;17:3878–85.
[27] Bours V, Bonizzi G, Bentires-Alj M, Bureau F, Piette J, Lekeux P, Merville M. NF-kappaB activation in response to toxical and therapeutical agents: role in inflammation and cancer treatment. Toxicology 2000;153:27–38.
[28] Chai J, Du C, Wu JW, Kyin S, Wang X, Shi Y. Structural and biochemical basis of apoptotic activation by Smac/DIABLO. Nature 2000;406:855–62.
[29] Jost CA, Marin MC, Kaelin Jr WG. p73 is a simian [correction of human] p53-related protein that can induce apoptosis. Nature 1997;389:191–4.
[30] Murakami Y, Sekiya T. Accumulation of genetic alterations and their significance in each primary human cancer and cell line. Mutat Res 1998;400:421–37.
[31] Ferreira CG, Span SW, Peters GJ, Kruyt FA, Giaccone G. Chemotherapy triggers apoptosis in a caspase-8-dependent and mitochondria-controlled manner in the non-small cell lung cancer cell line NCI-H460. Cancer Res 2000;60:7133–41.