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# Apaf-1 overexpression partially overcomes apoptotic resistance in a cisplatin-selected HeLa cell line

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**Abstract Inhibition of caspase-3-mediated apoptosis has been hypothesized to be associated with chemoresistance. Investigations of apoptosis revealed that cytosolic cytochrome *c* is associated with a complex of apoptotic protease activating factor-1 (Apaf-1), an adapter molecule, and caspase-9 to activate caspase-3. However, whether these apoptotic molecules are involved in acquired cisplatin resistance is not understood. The present work shows reduced activation of caspase-3 and apoptosis in a cisplatin-selected HeLa cell line. Ac-DEVD-CHO, a caspase-3 inhibitor, inhibited cisplatin-induced apoptosis about 60–70% in both cell lines. Ac-LEHD-CHO, a caspase-9 inhibitor or Ac-IETD-CHO, a caspase-8 inhibitor, inhibited cisplatin-induced caspase-3 activation and apoptosis similarly in both cell lines. In addition, cisplatin induced the activation of caspase-9, the upstream activator of caspase-3, in a dose-dependent manner, and the activation of caspase-9 was less induced in resistant cells. The accumulation of cytosolic cytochrome *c*, an activator of caspase-9, and the induction of the mitochondrial membrane-associated voltage-dependent anion channel were also reduced in cisplatin-resistant cells. However, the concentration of Bcl-2 family proteins in cisplatin-resistant cells was normal. The concentration of Apaf-1 was unaltered in both cell lines. Increasing the cellular concentration of Apaf-1 through the transient expression of the gene increased the induction of apoptosis in resistant cells, associated with enhanced activation of caspase-9, caspase-3 and DNA fragmentation factor. Regression analysis reveals that the modification factor, the ratio of the slope in the linear range of the dose–response curve with Apaf-1 to the slope without Apaf-1, is 1.5 and 4.75 in the HeLa and cisplatin-resistant HeLa cells, respectively. These results indicate that apoptosis and caspases are less induced in cisplatin-selected HeLa cells. They also suggest that ectopic overexpression of Apaf-1 may partially reverse the acquired cisplatin resistance. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

*Key words:* Apoptotic protease activating factor-1; Apoptosis; Cisplatin; Caspase-3; Cytochrome *c*

## 1. Introduction

*cis*-Diamminedichloroplatinum(II) (cisplatin) is an important chemotherapeutic agent for cancer treatment. The development of resistance to cisplatin is a clinical problem, and has led to investigation into the mechanism by which cells become resistant to cisplatin and ways to overcome cisplatin resistance [1–3]. The cellular response to diverse classes of stress inducers such as cisplatin includes, among other responses, activation of apoptosis [4,5]. Although significant biochemical changes have been described in cell lines made resistant to cisplatin [6–8], the role of apoptosis in cellular resistance to cisplatin remains incompletely described.

Two of the best characterized biochemical events during apoptosis are the activation of caspase-3 protease and the fragmentation of DNA into nucleosomal fragments. Existing investigations have suggested that mitochondria may play an important role in apoptosis by releasing cytochrome *c* [9,10]. Cytochrome *c* is associated with a complex of apoptotic protease activating factor-1 (Apaf-1) and caspase-9 and thus activates caspase-3 [11–13]. Activated caspase-3 cleaves poly(ADP-ribose) polymerase (PARP), protein kinase C δ, and other proteins [14,15]. Overexpression of the anti-apoptosis Bcl-xL protein interferes with this caspase activation by preventing the release of cytochrome *c* [9,10,16,17]. Earlier investigations have suggested that Bcl-xL (Bcl-2) may interact with Apaf-1 and inhibit Apaf-1-mediated activation of caspase-9 [18–20]. Nucleosomal DNA fragmentation occurs during apoptosis following activation of the DNA fragmentation factor (DFF), a heterodimeric protein that functions downstream of caspase-3. DFF includes a 40 kDa factor (DFF40) and its inhibitor DFF45, which is removed following caspase cleavage [21].

Several anti-cancer agents, including cisplatin, with diverse intracellular targets cause cytosolic accumulation of cytochrome *c*, and subsequent activation of caspase-3 [9,22,23]. However, the induction of Apaf-1, and its role in cellular resistance to cisplatin, remains obscure. The present investigation found that apoptosis and caspase activation are less induced in cisplatin-selected HeLa cells. Additionally, ectopic overexpression of Apaf-1 partially reverses the acquired cisplatin resistance.

## 2. Materials and methods

### 2.1. Cell lines and culture

Human cervix carcinoma HeLa and cisplatin-resistant HeLa cells (HeLa-CPR) [8] were maintained in Dulbecco's modified Eagle's me-

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*Abbreviations:* Apaf-1, apoptotic protease activating factor-1; Cisplatin, *cis*-diamminedichloroplatinum(II); PARP, poly(ADP-ribose) polymerase; VDAC, voltage-dependent anion channel; Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; Ac-LEHD-CHO, acetyl-Leu-Glu-His-Asp-aldehyde; Ac-IETD-CHO, acetyl-Ile-Glu-Thr-Asp-aldehyde


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dium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml; Gibco). The cell lines were maintained at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air. 1 μM cisplatin was added to the medium to maintain the resistant phenotype. Before any experiments, resistant cells intended as source materials were cultivated in media without cisplatin for 3 weeks.

*2.2. Western blot analysis*

Cells ($2\times10^6$) were treated with cisplatin, washed twice with phosphate-buffered saline (PBS) and lysed in a lysis buffer (RIPA, Tris–HCl 50 mM, pH 7.4, NP-40 1%, sodium deoxycholate 0.25%, NaCl 150 mM, EGTA 1 mM, phenylmethylsulfonyl fluoride (PMSF) 1 mM, protease inhibitor cocktail, $Na_3VO_4$ 1 mM and NaF 1 mM) on ice for 30 min. Insoluble material was removed by centrifugation at 14 000 rpm for 10 min at 4°C. Protein concentrations were measured using a protein assay kit (Bio-Rad, Hercules, CA, USA). Proteins were separated by SDS–PAGE, transferred onto PVDF membranes and incubated with antibodies reactive to CPP32/caspase-3, PARP, Apaf-1, caspase-9, voltage-dependent anion channel (VDAC), Bcl-xL, Bcl-2, Bax or DFF (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The antigen–antibody complexes were visualized by standard enhanced chemiluminescence reaction (Pierce, Rockford, IL, USA). Rabbit anti-caspase-9 was kindly provided by Dr. Xiaodong Wang, University of Texas Southwestern Medical Center.

*2.3. Analysis of apoptosis*

Cells growing in six-well plates were either left untreated or treated with cisplatin for 24 h. For inhibitor investigations, cells were preincubated with a caspase-3 inhibitor, Ac-DEVD-CHO, or caspase-9 inhibitor, Ac-LEHD-CHO, or caspase-8 inhibitor, Ac-IETD-CHO (Bachem, Switzerland), for 2 h. The cells were fixed with methanol and incubated with DAPI (4-diamidino-2-phenylindole) solution for 30 min in darkness. Floating cells from each well were also fixed and returned to the respective wells. All cells were analyzed using an Olympus microscope at 420 nm. Apoptotic cells exhibiting morphologic features of apoptosis, including chromatin condensation and nuclear fragmentation [24], were counted in six to eight randomly selected fields. Approximately 500 nuclei were examined for each sample, and the results are expressed as the number of apoptotic nuclei over the total number of nuclei counted.

*2.4. Determination of cytochrome c release*

Cells were washed twice with PBS, and suspended in buffer A (20 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM PMSF, and protease inhibitor cocktail) with 250 mM sucrose for 30 min on ice. The cells were then run through a Dounce homogenizer 15 times with a sand paper-polished pestle. Following centrifugation for 5 min at 4°C the supernatants were further centrifuged at $100\,000\times g$ for 30 min at 4°C. The resulting supernatant served as the soluble cytosolic fraction. Proteins were separated by SDS–PAGE, transferred onto PVDF membranes, and probed with anti-cytochrome *c* antibody (Santa Cruz Biotechnology).

*2.5. Construction and production of recombinant adenovirus*

Replication-deficient recombinant adenoviruses containing Apaf-1 were generated using the method described by He et al. [25]. Briefly, a 4.2 kb *Not*I/*Eco*RV fragment was isolated from pcDNA 3.1-Apaf-1 [11] and ligated with pAdTrack-CMV [25]. Following restriction enzyme mapping, linearized pAdTrack-CMV-Apaf-1 was cotransformed with pAdEasy1 into BJ5183 competent bacteria to generate recombinant pAdEasy1-GFP-Apaf-1. The genomic structure of pAdEasy1-GFP-Apaf-1 was confirmed by restriction mapping. Recombinant viruses were produced by transfecting linearized pAdEasy1-GFP-Apaf-1 into 293 cells using lipofectamine reagent (Gibco). For large-scale virus production, the recombinant viruses were harvested from 20 plates of 293 cells grown on a 10 cm dish following 48 h of infection and subjected to CsCl gradient centrifugation [26]. Following desalting by PD-10 Sephadex column (Pharmacia), the viruses were aliquoted and stored at −70°C. The titer of the viruses was determined using a spectrophotometer at 260 nm, with one $OD_{260}$ representing approximately $10^{12}$ pfu/ml.

*2.6. Overexpression of adenovirus-mediated Apaf-1*

Cells growing in the log phase were infected with either empty vector or Apaf-1 adenovirus. After 36 h, cells were either left untreated or treated with various concentrations of cisplatin for 24 h. Cells were washed with PBS and stained with DAPI to analyze the morphology and percentage of apoptotic cells. Samples were scrutinized under a microscope (Olympus, Japan). 50–60% of the cells became infected by the virus (multiplicity of infection, MOI, 500) as indicated by the green fluorescence.

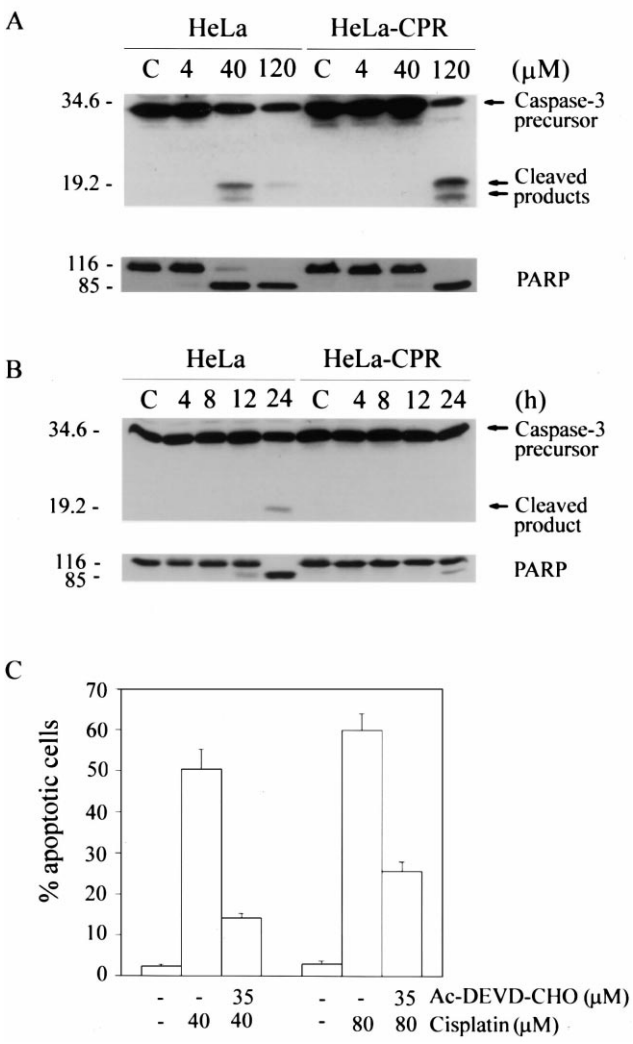


Fig. 1. Reduced activation of caspase-3 in resistant HeLa cells. A: Reduced activation of caspase-3 by cisplatin in resistant cells. HeLa and HeLa-CPR cells were either left untreated as controls (C) or treated with indicated concentrations of cisplatin for 24 h. Immunoblot analysis of the lysates was conducted with anti-caspase-3 or anti-PARP. The molecular weight markers (in kDa) are displayed on the left. B: Delayed activation of caspase-3 by cisplatin in resistant cells. Cells were treated with cisplatin (40 μM), harvested at indicated time points and immunoblotted with anti-caspase-3 or anti-PARP. C: Reduction of cisplatin-induced apoptosis with a caspase-3 inhibitor in both cell lines. Cells were pretreated with Ac-DEVD-CHO, a caspase-3 inhibitor, for 2 h and continuously treated together with indicated concentrations of cisplatin for 24 h. The percentage of apoptosis was determined by DAPI staining and microscopy. The plotted values denote the mean ± S.D. of three independent experiments.

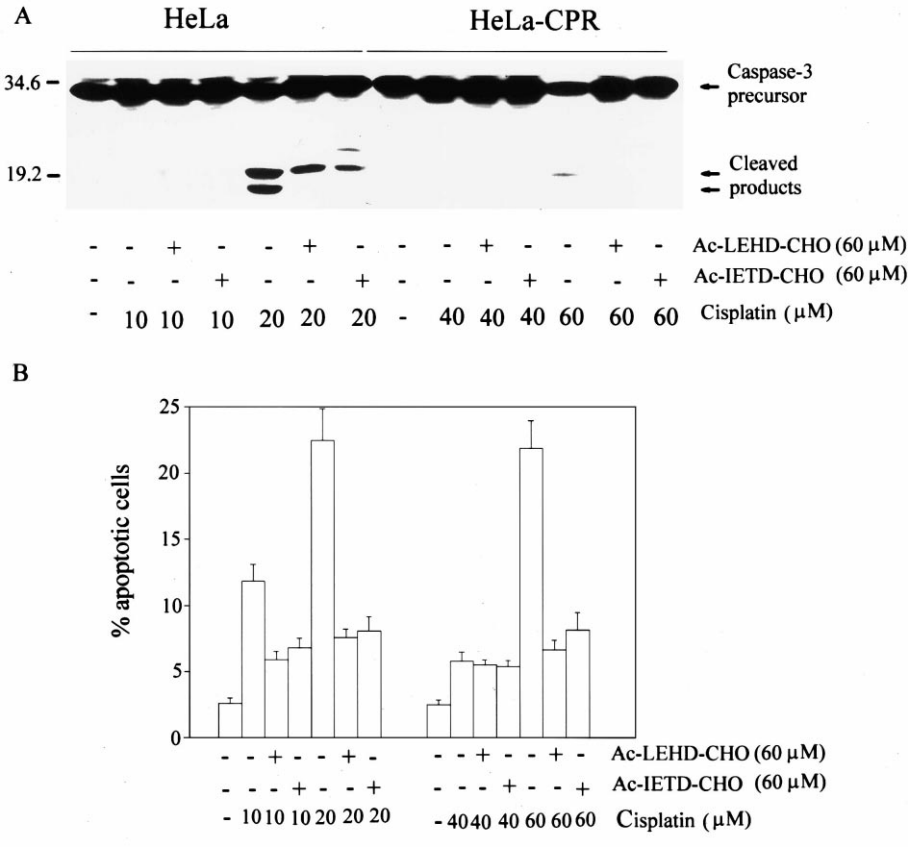


Fig. 2. Inhibition of cisplatin-induced caspase-3 activation and apoptosis by caspase-9 and -8 inhibitors. A: Inhibition of cisplatin-induced caspase-9 and -8 activation reduced the caspase-3 activation in both cells. HeLa and HeLa-CPR cells were either left untreated or pretreated with Ac-LEHD-CHO, a caspase-9 inhibitor, or Ac-IETD-CHO, a caspase-8 inhibitor, for 2 h and continuously treated together with indicated concentrations of cisplatin for 24 h. Immunoblot analysis of the lysates was conducted with anti-caspase-3. The molecular weight markers (in kDa) are displayed on the left. B: Inhibition of cisplatin-induced apoptosis by caspase-9 and -8 inhibitors in both cells. HeLa and HeLa-CPR cells were treated similarly as in A. The percentage of apoptosis was determined by DAPI staining and microscopy. The plotted values denote the mean ± S.D. of three independent experiments.

## 3. Results

### 3.1. Reduced activation of caspase-3 in cisplatin-resistant cells

Caspases are believed to be vital in mediating various apoptotic responses, including those mediated by cisplatin. To examine the role of apoptotic response in acquired cisplatin resistance, we first measured caspase-3 activation and apoptosis in a cisplatin-selected cell line. Caspase-3 was cleaved proteolytically from its inactive precursor form into active fragments by using cisplatin at a concentration of 40 μM in HeLa cells (Fig. 1A). Consistent with caspase-3 activation by cisplatin, the caspase-3 substrate protein, PARP, was also cleaved

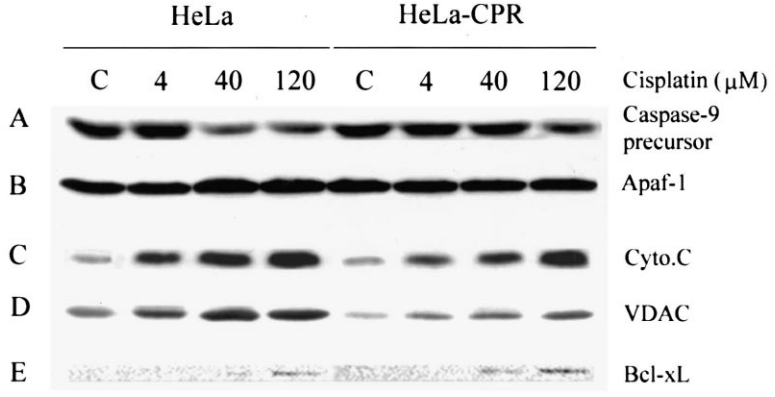


Fig. 3. Reduced induction of Apaf-1, cytochrome *c* release and caspase-9 activation in resistant HeLa cells. Cells were either left untreated (C) or treated with indicated concentrations of cisplatin for 24 h. Immunoblot analysis was conducted with anti-Apaf-1 (A), anti-caspase-9 (B), anti-VDAC (D) or anti-Bcl-xL (E). To study cytochrome *c*, cells were treated with cisplatin and harvested after 8 h. Cytosolic fractions were prepared as described in Section 2, and immunoblot analysis was performed with anti-cytochrome *c* (C).

from its 116 kDa to an 85 kDa fragment. However, the same treatment did not activate caspase-3 in resistant cells. At higher cisplatin concentrations, for example 120 μM, the caspase was effectively activated in resistant HeLa-CPR cells. Kinetic investigations also demonstrated that caspase-3 and PARP cleavage became active after 24 h at 40 μM of cisplatin in HeLa cells, but were either inactive or only minimally active in resistant cells (Fig. 1B). To determine whether caspase-3 activation is required for cisplatin-induced apoptosis, this pathway was inhibited using the caspase-3 inhibitor Ac-DEVD-CHO [27]. As illustrated in Fig. 1C, pretreatment of cells with a sublethal concentration of the caspase-3 inhibitor (35 μM) inhibited cisplatin-induced apoptosis about 60–70% in both cell lines. These experimental results reveal that although resistant cells display reduced apoptosis, the apoptosis machinery is probably not defective.

It is known that caspase-3 is a downstream effector of caspase-8 and caspase-9 in the apoptosis pathway [28–30]. To assess the upstream signal, resistant cells were compared with sensitive cells for their response to cisplatin with or without specific inhibitors for caspase-8, Ac-IETD-CHO, or caspase-9, Ac-LEHD-CHO. As shown in Fig. 2A, caspase-3 was activated in sensitive and resistant cells by 20 μM and 60 μM of cisplatin, respectively. In the presence of either inhibitor of caspase-8 or caspase-9, caspase-3 activation was reduced. This inhibitory effect was also observed in apoptosis. As shown in Fig. 2B, about 70% of cisplatin-induced apoptosis was inhibited in 20 and 60 μM-treated HeLa and HeLa-CPR cells, respectively, in the presence of either caspase-8 or -9 inhibitor. The results support the notion that the activation of caspase-3 upstream signal is also reduced in resistant cells, and that inhibition of caspase-8 or -9 activation blocks apoptosis sim-

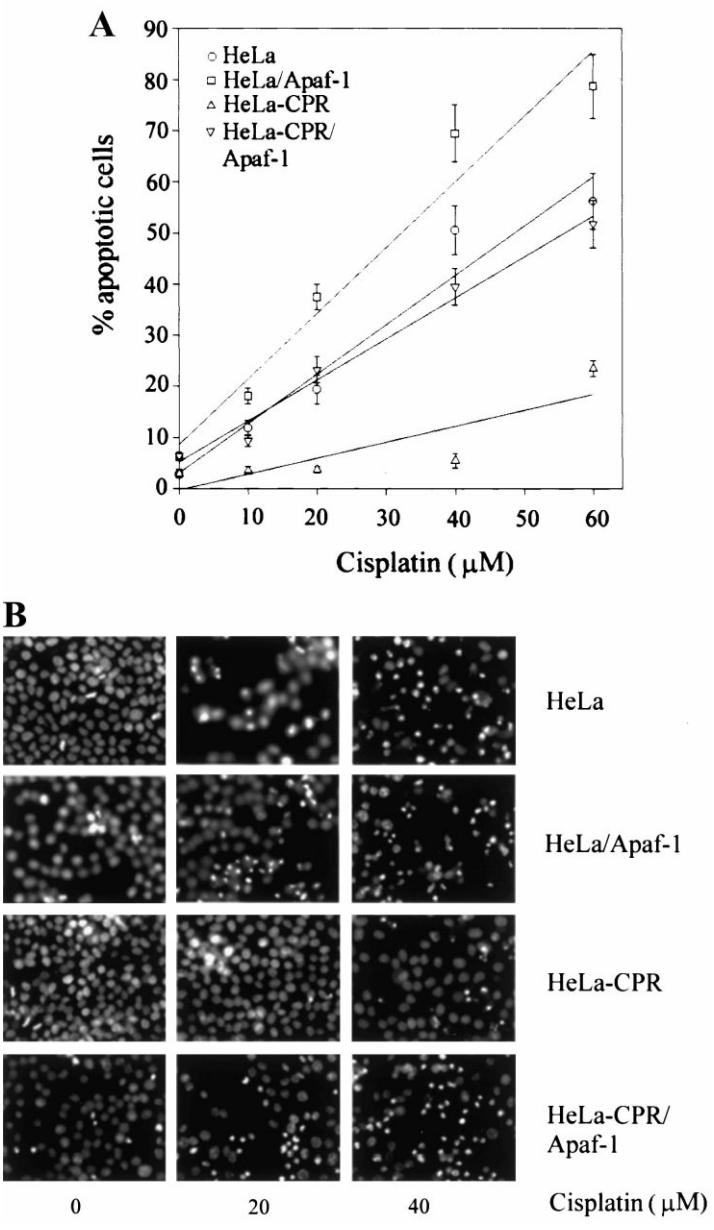


Fig. 4. Enhancement of cisplatin-induced apoptosis by overexpression of Apaf-1. The percentage of apoptotic cells was determined by DAPI staining of the infected cells. Cells infected with the empty vector or Apaf-1 adenovirus (MOI 500) were treated with cisplatin as indicated. The plotted values denote the mean ± S.D. of three independent experiments.

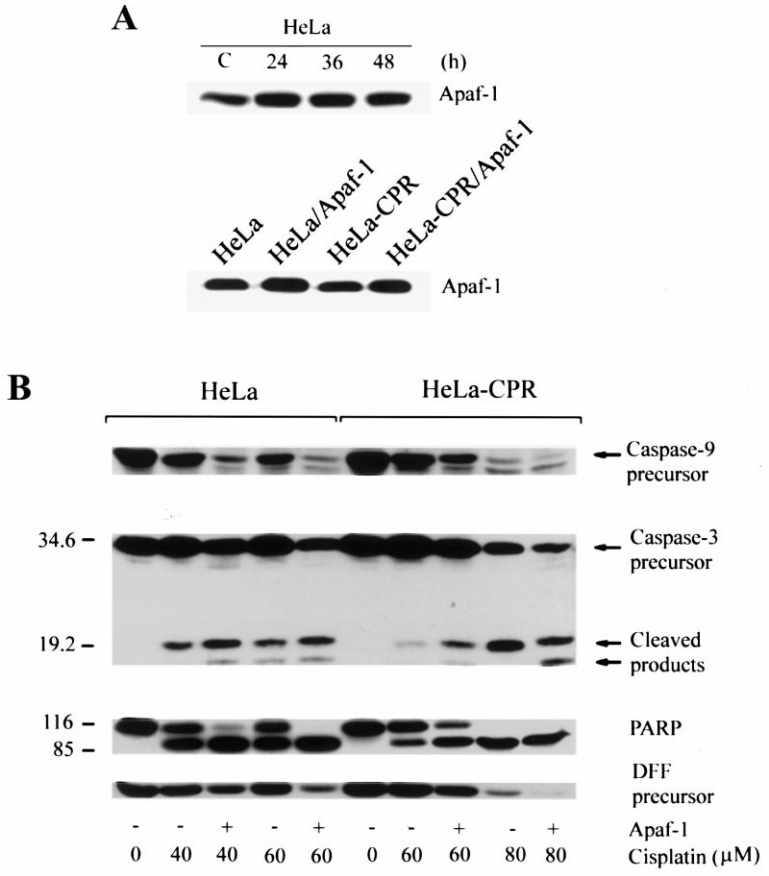


Fig. 5. Enhancement of cisplatin-induced activation of caspase-9, -3 and DFF by overexpression of Apaf-1. A: Overexpression of Apaf-1 in cells infected with adenovirus carrying the Apaf-1 gene. Cells were infected either with empty vector adenovirus (C) or with Apaf-1 adenovirus (MOI 500). Cells were harvested after the indicated times (top panel) or at 36 h (bottom panel) for immunoblot analysis with anti-Apaf-1. B: Enhancement of cisplatin-induced activation of caspase-9, -3 and DFF by overexpression of Apaf-1. Cells were infected with Apaf-1 adenovirus (MOI 500), and after 36 h they were treated with cisplatin as indicated for 24 h. Immunoblot analysis of the lysates was performed with anti-caspase-9, anti-caspase-3, anti-PARP or anti-DFF.

ilarly in both cell lines. These results also suggest that the apoptotic machinery is likely unaltered in resistant cells, compared to sensitive parental cells.

### 3.2. Reduced induction of cytochrome c release and caspase-9 activation in cisplatin-resistant cells

To reveal the upstream factor for reduced activation of caspase-3 in resistant cells, upstream caspases and their regulators were determined. Cytochrome *c* and dATP-dependent formation of the Apaf-1/caspase-9 complex has been demonstrated to activate caspase-3 [11–13]. In the present study, activation of caspase-9 was induced by cisplatin in a dose-dependent manner in both cell lines, but less in resistant cells (Fig. 3A). Here, only caspase-9 precursor was shown whose intensity decreased with drug concentration. Among the effectors of caspase-9 activation by the same treatment, the Apaf-1 level remained unchanged (Fig. 3B). Cytochrome *c* relocalized from mitochondria to the cytosol during apoptotic stimuli. To examine the role of cytochrome *c*, the accumulation of cytochrome *c* in partially fractionated cytosol was monitored. As confirmed in Fig. 3C, cisplatin caused cytochrome *c* accumulation in both cell types in a dose-dependent manner. However, induction in resistant cells was less than in sensitive cells. VDAC is an abundant protein in the outer mitochondrial membrane that forms a large voltage-gated pore in the planar lipid bilayers [31] and seems to serve, in combination with Bax, as a pathway for cytochrome *c* release from mitochondria. To test this hypothesis, we examined the induction of VDAC and its regulatory proteins Bcl-xL, Bcl-2 and Bax. Cisplatin was found to increase VDAC concentration in a dose-dependent manner in both sensitive and resistant cells (Fig. 3D). However, the induction was reduced in resistant cells. Although slight induction of Bcl-xL by cisplatin occurred in both cell types (Fig. 3E), only trace levels of Bcl-2 and Bax were induced, the protein levels remained largely unaltered (data not shown). Notably, the concentrations of these apoptotic proteins and regulators are the same in both cell lines. Thus, the resistant HeLa cells exhibited reduced activation of caspase-9 and reduced accumulation of cytochrome *c* release and VDAC. However, the concentration of Bcl-2 family proteins appeared normal in the resistant cells subjected to cisplatin.

### 3.3. Overexpression of Apaf-1 reverses the cisplatin-induced apoptotic resistance

It has been demonstrated that a reduced Apaf-1 level is associated with reduced apoptosis in cells, such as melanoma, resistant to chemotherapeutic drugs [32]. To determine the role of Apaf-1 in cisplatin resistance, we overexpressed Apaf-1 by adenovirus-transduced Apaf-1 cDNA in both cells.

Following cisplatin treatment, apoptotic cells with chromatin condensation and nuclear fragmentation were counted. Apaf-1 overexpression significantly enhanced cisplatin-induced apoptosis in parental and HeLa-CPR cells, and caused a greater enhancement of cisplatin-induced apoptosis by Apaf-1 in resistant cells (Fig. 4). Regression analysis reveals that the modification factor, the ratio of the slope in the linear range of dose–response curve with Apaf-1 to the slope without Apaf-1, is 1.5 and 4.75 in the HeLa and HeLa-CPR cells, respectively. Only cells infected with the virus (50–60%) as shown by green fluorescence were sampled.

Further, we examined whether overexpression of Apaf-1 could affect the cisplatin-induced activation of caspase-9, caspase-3 and DFF. Transient expression of Apaf-1 was detected in HeLa/Apaf-1 and HeLa-CPR/Apaf-1 cells. Kinetic expression of Apaf-1 indicated that the Apaf-1 level increased to a maximum at 24 h following infection (Fig. 5A). The basal level and the optimized expression level of Apaf-1, 36 h after virus infection, appeared 1.5–2-fold increased in both cell lines. The activation of caspase-9 increased with cisplatin concentration in both cells (Fig. 5B). Overexpression of Apaf-1 enhanced cisplatin-induced activation of caspase-9 by 1.5–2-fold (estimated by densitometry of the X-ray films) in both HeLa and HeLa-CPR cells. Caspase-3, a downstream effector caspase to caspase-9, was also enhanced by Apaf-1 overexpression as determined by the increased formation of cleaved products. The latter was confirmed by enhanced PARP cleavage by caspases. Since chromosomal fragmentation involves activation of DFF, the activation of DFF was also investigated. The intensity of the DFF precursor was slightly decreased, an indication of enhanced DFF activation, by cisplatin in both HeLa and HeLa-CPR cells. Overexpression of Apaf-1 enhanced cisplatin-induced activation of DFF by 1.5–2-fold (estimated by densitometry of the X-ray films) in both cells. These results support the notion that nuclear apoptosis is dependent on the activation of cytosolic caspases including caspase-3. These findings further strengthen the hypothesis that overexpression of Apaf-1 may partially reverse the acquired cisplatin resistance.

## 4. Discussion

In this report, we found reduced activation of caspase-3 and PARP cleavage in cisplatin-resistant cells compared to sensitive HeLa cells. Pretreatment of cells with the caspase-3 inhibitor Ac-DEVD-CHO inhibited cisplatin-induced apoptosis in both sensitive and resistant cells. Others have also shown that alteration in the expression of caspase-3 is associated with the development of cellular resistance to cisplatin [33]. These results support the notion that activation of caspase-3 is critical in the control of cisplatin-induced apoptosis in mammalian cells. Previous investigations have affirmed that various apoptotic stimuli other than cisplatin also activate caspase-3 and PARP cleavage [34–36]. Since inhibition of caspase-8 and -9 activation reduced the activation of caspase-3 and blocked apoptosis similarly in both cell lines, the apoptotic machinery in resistant cells is likely unaltered. Although reduced induction of caspase-3 mediated apoptosis in resistant cells, our findings suggest that the apoptotic machinery is likely unaltered.

Recent investigations confirmed that Apaf-1 exerts its effect by interacting with and promoting the oligomerization of procaspase-9, resulting in the sequential cleavage and activation of caspase-9 and caspase-3 [37]. The cellular concentration of Apaf-1 has also been reported to be an important molecular determinant of the threshold for apoptosis induced by paclitaxel, etoposide [38] and cisplatin [39,40]. Although the Apaf-1 level was not changed after cisplatin treatment as shown in HeLa cells, still the concentration of this molecule may be influential in apoptosis induced by drugs, and in the development of drug resistance. Unexpectedly, overexpression of Apaf-1 caused an equal effect on DFF activation, while it differentially modified apoptosis (i.e. chromosomal DNA fragmentation and chromatin condensation) by cisplatin in sensitive and resistant cells. Since DFF is a major activator for chromosomal DNA fragmentation [21], chromatin condensation can also be regulated by Apaf-1, and it is differentially regulated in the sensitive and resistant cells. These experimental results suggest that Apaf-1 may modify other factors involved in cisplatin-induced apoptosis besides DFF.

Diverse apoptosis-inducing agents, including UVB light, staurosporine, etoposide, actinomycin D and ara-C, can provoke the release of cytochrome *c* from mitochondria [10–12,32]. Inhibition of cytochrome *c* release is associated with acquired resistance to cytotoxic drugs [41]. Cells undergoing apoptosis were found to exhibit elevated cytochrome *c* in the cytosol, while overexpression of Bcl-2 prevented the efflux of cytochrome *c* from the mitochondria and the initiation of apoptosis by interacting with the mitochondrial channel VDAC [9,18,42]. Correlating with these reports, our results also displayed a significantly increased cytochrome *c* accumulation in cytosol following cisplatin treatment, and reduced induction of cytochrome *c* release in the cisplatin-resistant cells. Although cisplatin induced the expression of VDAC and Bcl-xL in sensitive and resistant HeLa cells, they only differed from the other samples in the induction of VDAC. However, both cell types expressed minimal levels of Bcl-2 and Bax, and their levels did not change significantly following cisplatin treatment (data not shown). Similarly, no change occurred in Bcl-2 following cisplatin treatment in either parental or cisplatin-resistant ovarian carcinoma cells [43]. The above results imply that controlling cytochrome *c* release is important for the resistance mechanism in HeLa cells.

Additionally, this investigation confirmed that overexpression of Apaf-1 enhances the susceptibility of cells to cisplatin-induced apoptosis and partially reverses cisplatin-induced apoptotic resistance. Although the basal level of Apaf-1 appeared to be similar in both cell lines, the modification of apoptosis is greater in resistant cells suggesting that overexpressed Apaf-1 itself may readily induce apoptosis in resistant cells through oligomerization [44]. Unlike apoptosis, which is determined only in infected cells, the fact that the modification in the activation of caspases in resistant cells is less compared to apoptosis may be due to mixed populations of uninfected and infected cells. Since the activation of Apaf-1 depends on the presence of dATP and cytochrome *c* [11,12], the two-fold reduced induction of apoptosis by cisplatin in resistant cells may be the result of reduced cytochrome *c* release. However, it has already been confirmed that DNA repair is enhanced two- to three-fold in the resistant cells [8,45]. Reduced cytochrome *c* release in cisplatin-resistant cells is unlikely to be influential besides enhancing DNA repair in acquired apoptotic resistance. Since our data suggest that the caspase signaling is unaltered in resistant HeLa cells,

reduced activation of caspase-9 and -3 in cisplatin resistance is probably signaled from upstream of the caspase pathway. Therefore, induction of cytochrome *c* release by cisplatin may be associated with DNA damage. Nevertheless, reduced induction of cytochrome *c* release in response to cisplatin damage can clearly cause reduced apoptosis through reduced caspase-9 and caspase-3 activity. This phenomenon may establish an opportunity for cells to develop resistance to cisplatin. Taken together, our results suggest that enhanced DNA repair in cisplatin-selected cells may be responsible for reduced apoptosis.

In summary, our results indicate that apoptosis and caspase activation are less induced in cisplatin-selected HeLa cells. Although Apaf-1 is not induced more in resistant cells, ectopic overexpression may partially reverse the acquired cisplatin resistance. Therefore, Apaf-1 can be a potential target for gene therapy against cancer. Although reduced induction of cytochrome *c* release and caspase activation may lead to apoptotic resistance, a role of Bcl-2 and Bcl-xL in regulating Apaf-1 activation cannot be ruled out. Further studies are needed to determine if our findings apply to other tumor cell models.

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