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Cisplatin-induced apoptosis by translocation of endogenous Bax in mouse collecting duct cells

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Abstract

cis-Platinum(II) (*cis*-diammine dichloroplatinum; cisplatin) is a potent antitumor compound that is widely used for the treatment of many malignancies. An important side-effect of cisplatin is nephrotoxicity, which results from injury to renal tubular epithelial cells and can be manifested as either acute renal failure or a chronic syndrome characterized by renal electrolyte wasting. Recently, apoptosis has been recognized as an important mechanism of cell death mediating the antitumor effect of cisplatin. This study was undertaken to examine the mechanisms of cell death induced by cisplatin in M-1 cells, which were derived from the outer cortical collecting duct cells of SV40 transgenic mice. Treatment of M-1 cells with high concentrations of cisplatin (0.5 and 1 mM) for 2 hr led to necrotic cell death, whereas a 24-hr treatment with 5–20 μM cisplatin led to apoptosis. Antioxidants protected against cisplatin-induced necrosis, but not apoptosis, indicating that reactive oxygen species play a role in mediating necrosis but not apoptosis induced by cisplatin and that the mechanism of cell death induced by cisplatin is concentration dependent. The low concentrations of cisplatin, which induced apoptosis in M-1 cells, did not affect the expression levels of Bcl-2-related proteins and did not activate c-Jun NH_2-terminal kinase (SAPK/JNK). Cisplatin induced the translocation of endogenous Bax from the cytosolic to the membrane fractions and, subsequently, the release of cytochrome *c*. Overexpression of Bcl-2 blocked cisplatin-induced apoptosis and Bax translocation. These observations suggest that the subcellular redistribution of Bax is a critical event in the apoptosis induced by cisplatin.

Keywords: Cisplatin; Collecting duct; Apoptosis; Bax translocation; Bcl-2

1. Introduction

cis-Platinum(II) (*cis*-diamminedichloroplatinum; cisplatin) is widely used for the treatment of many malignancies, including testicular, ovarian, head and neck, bladder, esophageal, and small cell lung cancer [1,2]. An important side-effect of cisplatin is nephrotoxicity, which results from injury to renal tubular epithelial cells and can be manifested as either acute renal failure or a chronic syndrome characterized by renal electrolyte wasting [3].

The cytotoxicity of cisplatin is believed to be due to the formation of DNA adducts, which include DNA-protein cross-links, DNA monoadducts, and interstrand and intrastrand DNA cross-links [4,5]. Further studies demonstrated that the cytotoxicity of cisplatin is probably due to a combination of insults, including peroxidation of the cell membrane [6], mitochondrial dysfunction [7], inhibition of protein synthesis [8], and DNA injury [9]. The presence of necrosis, characterized by swelling of mitochondria and other intracellular organelles [10,11], has clearly been demonstrated in tissue sections of kidneys from patients with cisplatin nephrotoxicity [3,12]. Apoptosis is a form of cell death with morphological features quite distinct from necrosis [10,11,13]. In contrast to necrosis, apoptosis is characterized by both cytosolic and nuclear shrinkage. The nuclear chromatin in the apoptotic cell undergoes con-

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Abbreviations: ROS, reactive oxygen species; SAPK/JNK, stress-activated protein kinase/c-Jun NH_2-terminal kinase; RT–PCR, reverse transcription–polymerase chain reaction; ECL, enhanced chemiluminescence; LDH, lactic dehydrogenase; DPPD, diphenyl-*p*-phenylene-diamine; DFO, deferoxamine; DMTU, dimethylthiourea; and BHA, butylated hydroxyanisole.

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densation, whereas the plasma membrane and mitochondria remain morphologically intact. Recently, apoptosis was recognized as an important mechanism of cell death, mediating the antitumor effect of cisplatin, as well as other chemotherapeutic agents [14]. Cisplatin has been reported to induce apoptosis in renal epithelial cells [15,16]. A recent study suggests that apoptotic cell death may play an important role in the development of cisplatin-induced acute renal failure [17].

A number of stimuli have been shown to induce necrosis as well as apoptosis [7,18]. It has been suggested that the mechanism of cell death in response to injury is, in part, a function of the concentration of the noxious stimulus to which cells are exposed [19,20]. Exposure to high concentrations of a toxin or to severe depletion of cytosolic ATP leads to rapid metabolic collapse, and necrosis [14,20]. In contrast, lower concentrations of the same agents have been shown to cause apoptosis [14,20]. In renal epithelial cells, high concentrations of cisplatin led to necrotic cell death within a few hours, whereas low concentrations led to apoptosis over several days [15].

The mechanisms of cisplatin-induced cytotoxicity are not clear. Recent reports showed that cisplatin-induced cytotoxicity is related to the generation of ROS, and that antioxidants protect against cisplatin-induced cell injury and acute renal failure [21]. It has been shown that ROS play a role in mediating apoptosis but not in necrosis induced by cisplatin in renal proximal tubule cells [21], and that superoxide radicals, not hydroxyl radicals, are involved in acute cell injury by cisplatin in rabbit renal cortical slices [22]. However, Kruidering *et al.* [23] reported that ROS formation occurs during cisplatin-induced toxicity in porcine renal proximal tubular cells, but that it is not the direct cause of cell death. Roller and Weller [24] showed that antioxidants specifically inhibit cisplatin toxicity in human malignant glioma cells in the absence of drug-induced free radical formation. Thus, the role of ROS in cisplatin-induced cytotoxicity is not clear.

Activation of p53 is involved in cisplatin-induced apoptosis via the increased expression of Bak and Bax [25] or activation of the CD95 receptor/ligand system [26]. However, cisplatin has been reported to induce apoptosis in testicular cancer cells by a p53-independent mechanism, and its action was not correlated with the expression level of Bcl-2 and Bax [27]. Cisplatin still induced apoptosis in Fas-resistant Jurkart cells [28] and Hep 3B cells [29], suggesting that Fas-independent mechanisms are also involved in cisplatin-induced apoptosis.

The role of SAPK/JNK in cisplatin-induced apoptosis is still controversial. SAPK/JNK plays an important role in cisplatin-induced apoptosis in thermosensitive parental cells [30], but activation of SAPK/JNK by cisplatin had a protective effect against apoptosis in glioblastoma cells [31]. Futhermore, its effect on cisplatin-induced apoptosis has not been examined in kidney epithelial cells.

This study was undertaken to elucidate the mechanisms of cisplatin-induced cytotoxicity in M-1 cells. M-1 cells, which were derived from the outer cortical collecting ducts of mice transgenic for the early region of simian virus SV40 (large T antigen) [32], were used for these experiments because they retain many of the differentiated characteristics of collecting ducts [33,34].

2. Materials and methods

2.1. Cell culture

M-1 cells were obtained from the American Type Culture Collection, and were grown in DMEM/F12 (Life Technologies Inc.) at 37° in a 5% CO_2 atmosphere.

2.2. DNA fragmentation

Low molecular weight genomic DNA was extracted as follows. The cell pellet was lysed using 0.5 mL of extraction buffer [0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris (pH 7.4)] for 20 min on ice. The low-molecular-weight DNA-containing soluble fraction was isolated by centrifugation at 15,000 *g* for 10 min at 4°, phenol-chloroform extracted three times, ethanol precipitated, resuspended in Tris/EDTA (pH 8.0), containing 20 μg/mL of RNase A, and incubated at 37° for 1 hr. DNA was run on a 2% agarose gel and was visualized by ethidium bromide staining.

2.3. Determination of cell viability

After treating cells with cisplatin, cells were trypsinized, placed in cell suspension in PBS, and stored on ice until a cell count could be performed. A small volume of 0.4% trypan blue (Sigma) was added to an equal volume of cell suspension, and the mixture was incubated for 10 min. Cell death was determined by the presence of cytoplasmic trypan blue. The total cell number per culture bottle and the percentage of nonviable cells were determined using a hemocytometer under light microscopy.

2.4. RT–PCR

Total RNA was isolated from cells using RNAzol B (Tel Test Inc.), reverse transcribed into first stand cDNA using oligo dT primers, and amplified by 35 cycles (94°, 1 min; 50°, 1 min; 72°, 1 min) of PCR using 20 pmol of specific primers. Upon completion of the PCR, products were examined on a 2% agarose gel. β-Actin primers were used as an internal standard. The primers used for PCR were as follows:

2.5. Western blot analysis

Confluent monolayers of cells, treated as described in the legend of Fig. 5, were scraped from culture dishes into

microfuge tubes. The cells were lysed at 4° in a solution containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL of leupeptin, 10 μg/mL of aprotinin, and 1 mM sodium orthovanadate. Lysates were centrifuged at 10,000 *g* for 10 min at 4° to remove insoluble material, and total protein concentration in the supernatants was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories) using γ-globulin as the standard. Proteins (200 μg) were suspended in 5 × sample loading buffer [500 mM Tris, 5% 2-mercaptoethanol, 10% glycerin, 2.5% SDS, 0.0125% bromphenol blue (pH 6.8)], and resolved on two 12% SDS-polyacrylamide gels. One gel was electrotransferred to nitrocellulose membranes (Hybond-ECL, Amersham Life Science) and probed with polyclonal antibodies (Bcl-X, Bak, Bax, and Bcl-2; Santa Cruz Biotechnology). The other gel was stained with Coomassie Blue to confirm equal loading of protein amounts to each lane. Immunoreactive bands were detected using horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibodies (Amersham Life Science) and were visualized by ECL (Amersham Life Science). Negative controls were performed by using the primary antibodies preadsorbed with a 50-fold molar excess of immunizing peptide. Results were quantified using a scanning densitometer (Bio-Rad Laboratories). The data were presented as a percentage of the control protein levels within each group.

2.6. SAPK/JNK assays

M-1 cells were treated with cisplatin (5 μM) in serum-free medium and were lysed at 4° in lysis buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-mercaptoethanol, 1 mM sodium orthovanadate, 1 μg/mL of leupeptin, and 1 mM phenylmethylsulfonyl fluoride]. Lysates were centrifuged at 13,000 *g* for 10 min at 4°, and equalized for protein. For the SAPK/JNK assay, the supernatant was incubated with an N-terminal c-Jun [1–9] fusion protein bound to glutathione Sepharose beads for selectively pulling down SAPK/JNK from cell lysates [35]. The immune complexes were pelleted and washed twice in immunoprecipitation buffer and then twice in kinase buffer. The kinase reaction for the JNK assay was carried out in kinase buffer [25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, and 10 mM $MgCl_2$] containing 100 μM ATP, and c-Jun phosphorylation was measured using a phospho-specific c-Jun antibody. The antigen–antibody complexes were visualized by chemiluminescence (ECL detection system, Amersham Life Science).

2.7. Bax translocation

For immunoblotting, cytosolic and membrane fractions were prepared by selective plasma membrane permeabilization with digitonin [36]. After treatment with 0.05% digitonin in isotonic sucrose buffer (250 mM sucrose, 10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, and 1 mM EGTA; pH 7.1), for 1 min at room temperature, the cells were centrifuged at 15,000 *g* for 10 min at 4°. Supernatants (cytosolic fraction) were removed and saved. The insoluble pellets were extracted with ice-cold 0.5% Triton X-100 in isotonic sucrose buffer for 10 min to release membrane and organelle-bound soluble proteins including mitochondrial Bax. The samples were centrifuged as above, and membrane fractions were collected. Protease inhibitors were included in all solubilization buffers. Protein in the resultant supernatants was resolved by SDS–PAGE for western blot analysis using appropriate antibodies. To determine the degree of cross-contamination between cytosolic fractions and membrane fractions, LDH activity was measured using an LDH kit (Iatron Lab.), and cytochrome *c* oxidase activity was measured by the assay procedure of Wharton and Tzagoloff [37].

2.8. Cytochrome c release

After incubation, cells were washed twice with ice-cold PBS, resuspended in 100 μL of extraction buffer (50 mM HEPES, 220 mM mannitol, 68 mM sucrose, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride), and allowed to swell on ice for 30 min [38]. Cells were homogenized by passing the suspension through a 25-gauge needle (10 strokes). Homogenates were centrifuged in a Beckman Airfuge at 100,000 *g* for 15 min at 4°, and supernatants were harvested and stored at –70° until analysis by gel electrophoresis. Ten micrograms of cytosolic protein, as determined by the Bio-Rad protein assay, was loaded onto a 15% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose sheets, which were blocked with 5% nonfat dry milk in Tris-buffered saline and 0.05% Tween 20 (TBST), and probed in TBST with anti-cytochrome *c* monoclonal antibody (1:1000). After incubation with a 1:2000 dilution of anti-rabbit IgG, horseradish peroxidase linked whole antibody, blots were developed by ECL.

2.9. Development of stable cell lines

To generate stably transfected lines, M-1 cells were transfected with a *bcl-2* expression construct by lipofection (Lipofectamine Plus; Life Technologies). Cells were plated at 10^5 cells/well in a 6-well plate and transfected the next day with 2 μg of DNA, 6 μL of PLUS reagent, and 8 μL of lipofectamine/well. After 48 hr, cells were replated into a 10-cm dish and selected with medium containing 400 μg/mL of geneticin. After selection with the antibiotic for 10–14 days, individual resistant colonies were picked and expanded. At least 12 clones were picked for each transfection, and the expression of protein was assessed by western blotting of cell lysates using Bcl-2 antibody.

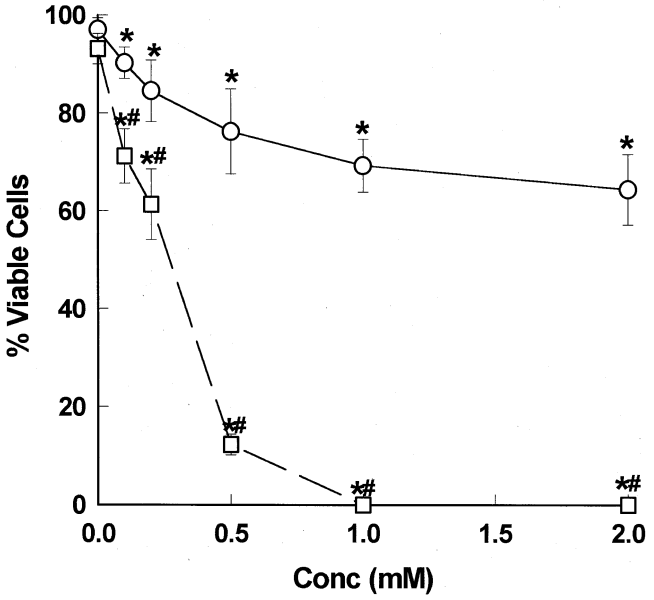

Fig. 1. Effect of various concentrations of cisplatin on M-1 cell viability. Cells (1×10^6) were treated with various concentrations of cisplatin for 2 (μ) and 24 hr (θ) at 37°, and cell viability was measured by the trypan blue exclusion assay as described under "Materials and methods." Data represent means ± SEM (N = 4). Key: (*) $P < 0.05$ compared with control values, and (#) $P < 0.05$ compared with the data from the 2-hr treatment with cisplatin.

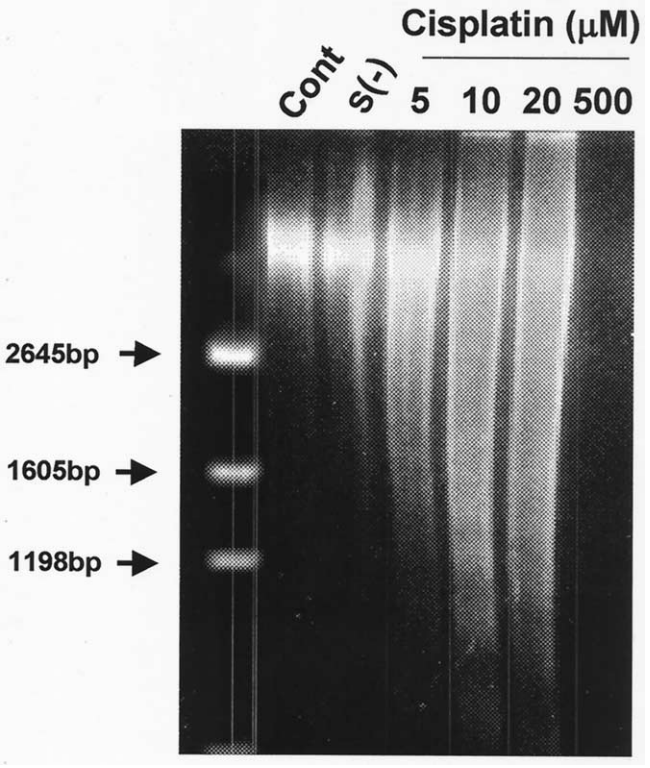

Fig. 2. Effect of cisplatin on DNA fragmentation in M-1 cells. M-1 cells were treated with various concentrations of cisplatin for 24 hr, and isolated genomic DNAs were analyzed in 2% agarose gel, as described under "Materials and methods."

3. Results

3.1. Cisplatin-induced death of M-1 cells

Exposure of M-1 cells to various concentrations of cisplatin for 2 and 24 hr in serum-free medium led to a concentration-dependent decrease in cell viability, as assessed by trypan blue exclusion. Treatment with 0.1 mM cisplatin decreased cell viability significantly ($P < 0.05$) (Fig. 1). The 24-hr treatment with cisplatin was significantly more cytocidal than the 2-hr treatment, and 100% of the cells were dead after 24 hr of treatment with 1 and 2 mM cisplatin.

To identify whether cisplatin induces apoptosis, degradation of genomic DNA was examined by agarose gel electrophoresis. Cells were treated with cisplatin for 24 hr in serum-free medium. Removal of serum from the culture medium for 24 hr did not induce a ladder pattern of DNA indicative of internucleosomal cleavage. "Low" concentrations of cisplatin (5–20 μM) induced DNA fragmentation, whereas DNA electrophoresis of M-1 cells exposed to 500 μM cisplatin did not show any DNA fragmentation in spite of massive cell death (Fig. 2).

3.2. Effects of antioxidants on cell viability and apoptosis in M-1 cells

It has been suggested that the cytotoxicity of cisplatin is related to the generation of ROS [21]. To examine this possibility, the effects of various antioxidants on cell viability were measured. As shown in Fig. 3, 10 μM DPPD, and 50 μM DFO decreased cell death induced by 0.5 mM cisplatin treatment for 2 hr, but DMTU (30 μM) had no effect.

Next, we determined the effects of antioxidants on cisplatin-induced DNA fragmentation. In this experiment M-1 cells were treated with 5 μM cisplatin for 24 hr. In contrast with their protective effects on necrotic cell death, 50 μM DFO, 30 μM DMTU, and 100 μM BHA failed to decrease cisplatin-induced apoptosis (Fig. 4). To further examine the effects of antioxidants on cell injury induced by 24-hr treatment with 5 μM cisplatin, we measured the viability of M-1 cells by trypan blue exclusion. As shown in Fig. 4, cisplatin treatment caused the death of 25% of the cells. The cells were not protected from death by the addition of DMTU or DFO. In this experiment, we did not use DPPD, because a 24-hr treatment with DPPD induced the death of most of the cells (data not shown).

3.3. Roles of Bcl-2-related proteins and SAPK/JNK in cisplatin-induced apoptosis

To determine whether the expression levels of the Bcl-2-related proteins were changed during apoptosis, M-1 cells were treated with cisplatin for 24 hr in serum-free medium,

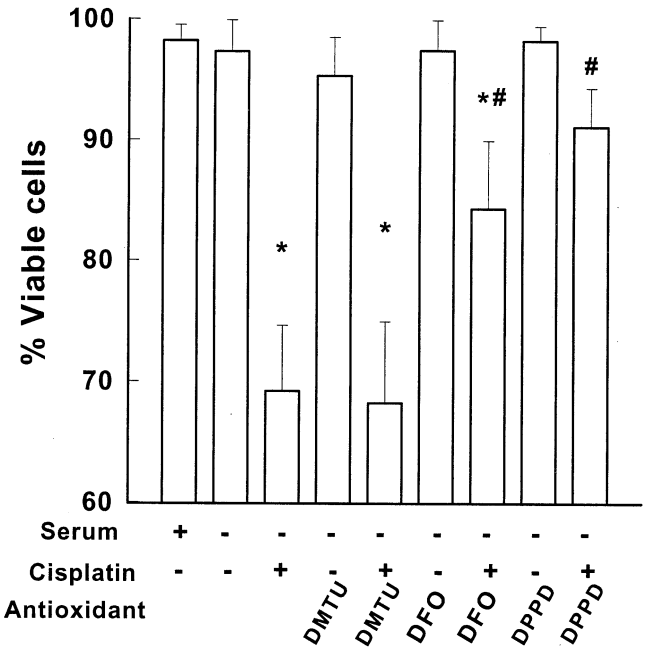

Fig. 3. Effects of antioxidants on cisplatin-induced cytotoxicity in M-1 cells. M-1 cells were treated for 2 hr with 0.5 mM cisplatin, and cell viability was measured by the trypan blue exclusion assay as described under "Materials and methods." The concentrations of antioxidants used for the experiment were: 30 μM DMTU, 50 μM DFO, and 10 μM DPPD. Data represent means ± SEM (N = 4). Key: (*) $P < 0.05$ compared with control values, and (#) $P < 0.05$ compared with the data from cisplatin-treated cells.

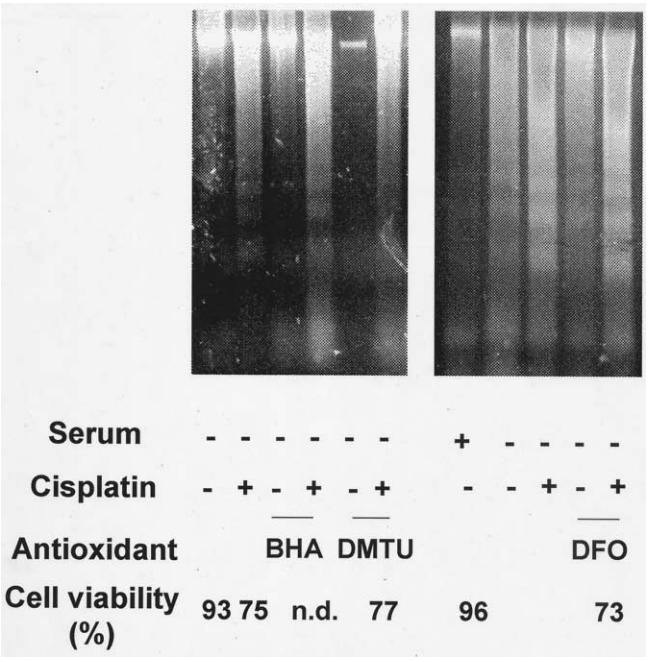

Fig. 4. Effects of antioxidants on cisplatin-induced DNA fragmentation. M-1 cells were treated with 5 μM cisplatin for 24 hr in the absence or presence of antioxidants, and isolated genomic DNAs were analyzed on 2% agarose gels, as described under "Materials and methods." The concentrations of antioxidants used for the experiment were: 30 μM DMTU, 50 μM DFO, and 100 μM BHA. At the bottom of the panel, the effects of antioxidants on cisplatin-induced cell death are shown. Data represent the means of three separate experiments.

and the protein levels of Bak, Bax, and Bcl-X_L were analyzed by western blots. Western blot analysis revealed that Bak, Bax, and Bcl-X_L were expressed in M-1 cells, and that the protein levels of Bak, Bax, and Bcl-X_L were not altered by treatment of M-1 cells with 5 μM cisplatin for 24 hr (Fig. 5). The preadsorption of primary antibodies with the corresponding synthetic peptide resulted in ablation of the bands shown in Fig. 5 (data not shown).

To determine whether the mRNA levels of Bcl-2 or Bcl-X_L were changed during cisplatin treatment, RT–PCR was done using total RNA isolated from control cells and cisplatin-treated cells. Treatment with cisplatin was performed as described in the legend of Fig. 5. To confirm whether equal amounts of RNA were used for the RT–PCR, β-actin mRNA, which remained constitutive during *in vitro*-induced apoptosis, was also amplified. The mRNA levels of Bcl-X_L and Bcl-2 were not changed in cisplatin-treated cells (Fig. 6).

To further test the role of Bcl-2 in cisplatin-induced apoptosis, cells overexpressing Bcl-2 were established. Figure 7A shows a marked increase in the expression of Bcl-2 in a transfected clone (BT). Cisplatin-induced apoptosis was compared between vector transfected cells and Bcl-2 overexpressing clones. As shown in Fig. 7B, cisplatin-induced DNA fragmentation was completely inhibited in a BT clone compared with vector-transfected cells (M-1).

Activation of the SAPK/JNK pathway is an important factor in apoptosis [39]. Therefore, to identify the involvement of the pathway in cisplatin-induced apoptosis of M-1 cells, SAPK/JNK activity was measured. SAPK/JNK activity was not altered by cisplatin treatment of M-1 cells in serum-free medium over time (Fig. 8). We also determined JNK activation after 6 and 12 hr of treatment, but did not observe SAPK/JNK activation at that time (data not shown). Removal of serum from the culture medium alone did not affect SAPK/JNK activity. As a positive control, the effect of an increase in medium osmolarity on SAPK/JNK was examined. Medium osmolarity was increased to 500 mOsM by adding 100 mM NaCl to the medium. Exposure of M-1 cells to a 500 mOsM medium for 15 and 30 min increased SAPK/JNK activity significantly.

3.4. Cisplatin-induced translocation of Bax from cytosol to mitochondria

Bax is thought to be present in the cytosol in a monomeric form since enforced dimerization leads to its mitochondrial translocation [40]. Previous studies have indicated that Bax undergoes conformational changes in response to death signals from cytotoxic insults, such as survival factor withdrawal resulting in Bax homodimerization and mitochondrial membrane insertion [40]. To determine Bax translocation, we prepared cytosolic and mem-

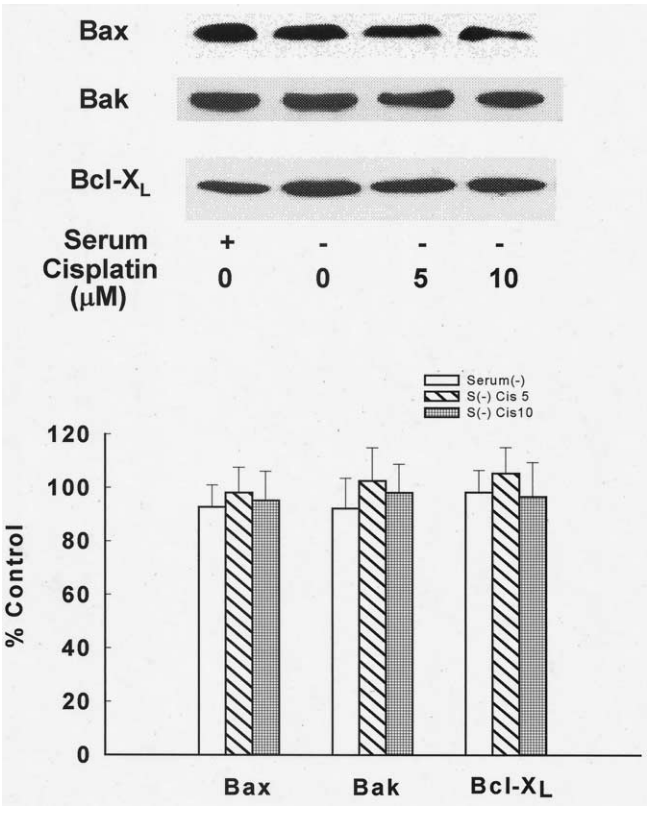

Fig. 5. Immunoblot analysis of Bcl-2-related proteins in M-1 cells. Proteins were isolated from control cells (lane 1) or cells grown in serum-free medium in the absence (lane 2) and presence of 5 μM (lane 3) or 10 μM (lane 4) cisplatin for 24 hr. Top, representative western immunoblots. Bottom, quantitative results. Data are expressed as a percentage of the control levels for four independent experiments.

brane-bound fractions from control cells or from M-1 cells treated with cisplatin in serum-free medium. Digitonin was used to selectively permeabilize the plasma membranes of cells to obtain the cytosolic fraction [36]. Digitonin-insoluble residues were further extracted with Triton X-100 to release membrane-associated and organelle-bound proteins. To determine the purity of the fractions to be used in the experiment, LDH and cytochrome *c* oxidase activities, as marker enzymes of cytosol and mitochondria, respectively, were determined. LDH activities in the cytosolic and membrane fractions were 280 ± 17 and 39 ± 3 U/mg protein, respectively, and cytochrome *c* oxidase acitivities were 0.01 ± 0.002 and 0.14 ± 0.02 U/mg protein. To determine

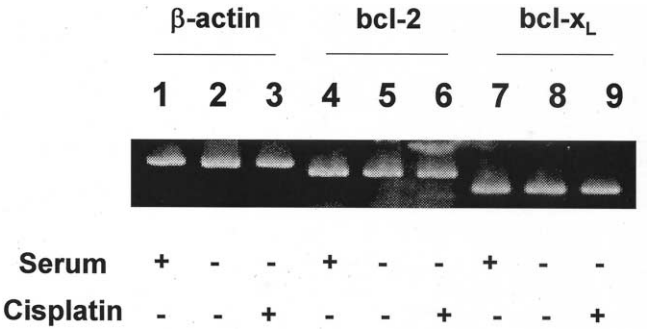

Fig. 6. Effect of cisplatin on Bcl-X_L and Bcl-2 mRNA expression in M-1 cells. RT–PCR products were generated using Bcl-X_L or Bcl-2 specific primers and analyzed on 2% agarose gels. To compare RNA integrity and quantity between samples, amplification products using the primers specific for β-actin are shown. Total RNA was isolated from control cells (lanes 1, 4, and 7), and cells grown in serum-free medium in the absence or presence of cisplatin for 24 hr.

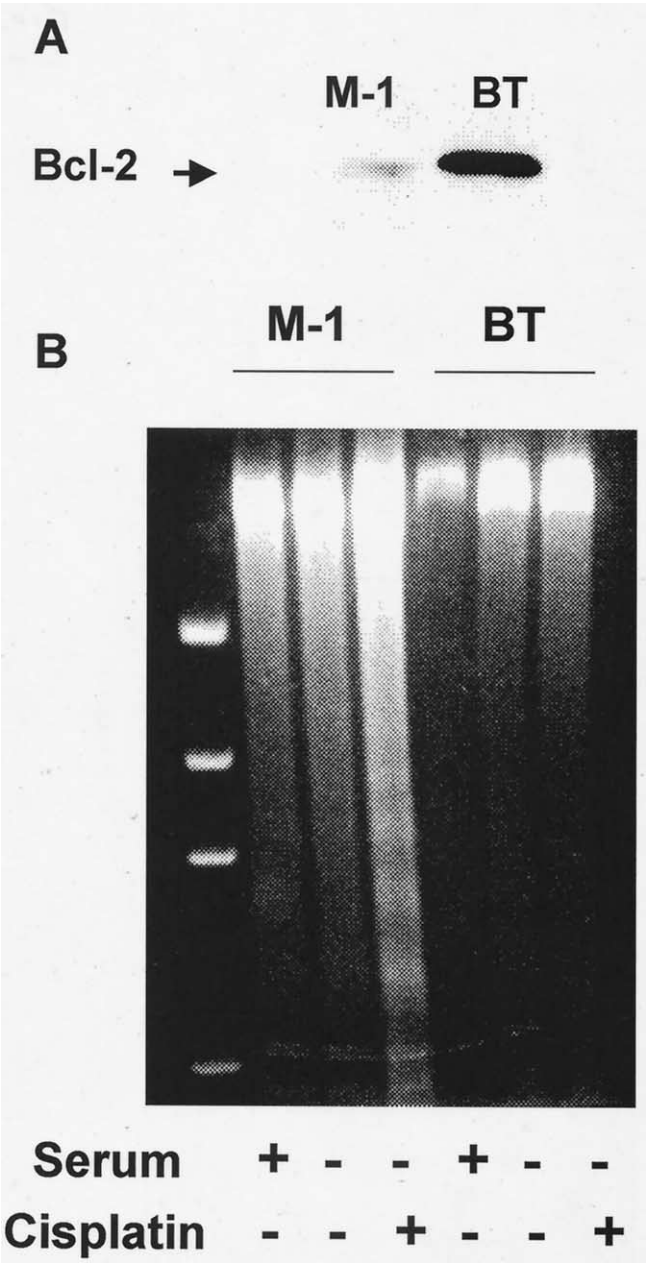

Fig. 7. Effect of Bcl-2 overexpression on cisplatin-induced apoptosis. (A) Western blot of M-1 cells using antibody against Bcl-2. BT = a transfected Bcl-2 overexpressing clone. M-1 = cells transfected with vector alone. Control protein samples were obtained from vector-transfected M-1 cells. (B) DNA fragmentation of Bcl-2 overexpressing clones. Vector-transfected M-1 cells or Bcl-2 overexpressing cells were cultured in the presence of 5 μM cisplatin. Cells were collected, and genomic DNAs were analyzed on 2% agarose gels.

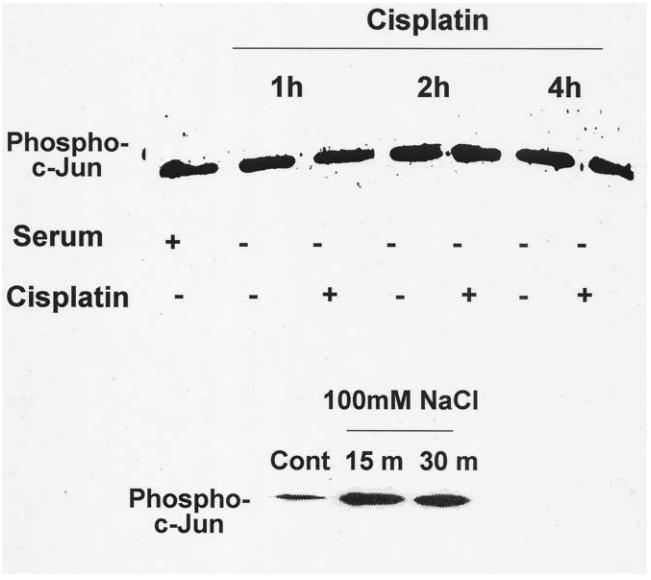

Fig. 8. Effect of cisplatin on SAPK/JNK activation. M-1 cells were treated with 5 μM cisplatin in serum-free medium for the times indicated on the figure. SAPK/JNK activity was analyzed by the method described under "Materials and methods." As a positive control, SAPK/JNK activity was also measured in M-1 cells exposed to hypertonic medium for the times indicated. Medium osmolarity was raised by the addition of 100 mM NaCl.

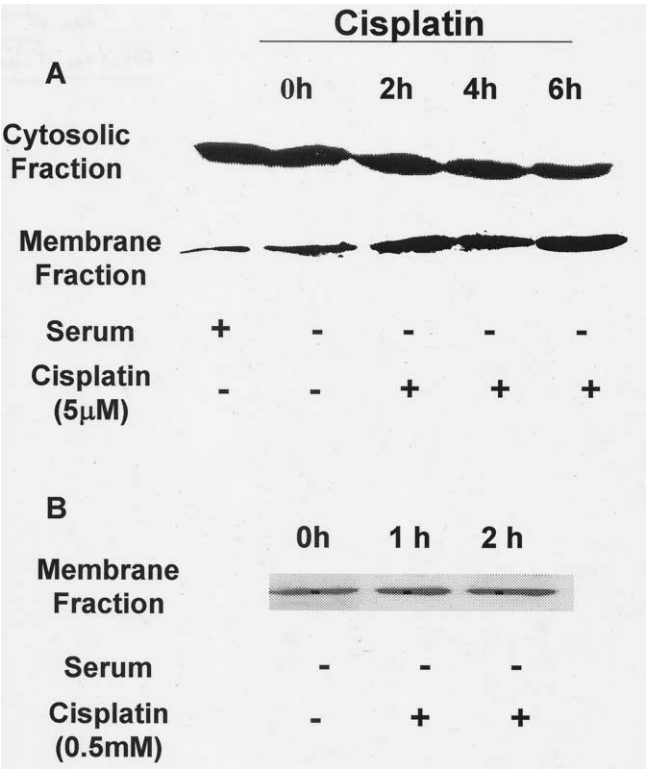

Fig. 9. Immunoblot analysis of Bax translocation to membrane fractions. (A) Membrane fractions were isolated from control cells and cells grown in serum-free medium for 2, 4, or 6 hr in the absence and presence of 5 μM cisplatin. (B) Membrane fractions were isolated from control cells and cells grown in serum-free medium for 1 or 2 hr in the absence and presence of 0.5 mM cisplatin. The levels of Bax in the mitochondrial membrane fractions were analyzed by immunoblot analysis (as described under "Materials and methods").

the time course of Bax translocation, cells were treated with cisplatin (5 μM) for 2, 4, and 6 hr, and the expression levels of Bax in cytosolic and membrane fractions were analyzed by SDS–PAGE and western blotting. Cisplatin induced Bax translocation to membrane fractions with a concomitant decrease of its expression in cytosolic fractions (Fig. 9). The 2-hr treatment with a high concentration of cisplatin (0.5 mM), which induced significant cell death, did not induce Bax translocation. DPPD (10 μM) protected against necrotic cell death induced by treatment with 1 mM cisplatin for 2 hr. Therefore, we examined whether DPPD prevents cisplatin-induced Bax translocation. The addition of 10 μM DPPD did not affect Bax translocation induced by 5 μM cisplatin (Fig. 10). To examine whether Bax translocation is a general phenomenon in the apoptotic death of M-1 cells, Bax translocation was determined in cells that were cultured in petri dishes for 6 hr. Park *et al.* [41] showed that detachment of M-1 cells from culture dishes induces apoptosis. Suspension culture did not induce Bax translocation. We showed that Bcl-2 overexpressing cells were resistant to cisplatin-induced apoptosis. Therefore, the effect of Bcl-2 overexpression on cisplatin-induced Bax translocation was determined. As shown in Fig. 11, 5 μM cisplatin induced Bax translocation to M-1 membrane fractions, whereas it failed to induce Bax translocation to membrane fractions in Bcl-2 overexpressing BT cells.

3.5. Effect of cisplatin on cytochrome c *release*

To determine whether Bax translocation was accompanied by the release of cytochrome *c*, M-1 cells were treated with 5 μM cisplatin for 6 and 24 hr in serum-free medium, and the levels of cytochrome *c* in the cytosolic fractions of M-1 cells were analyzed by western blot. As shown in Fig. 12, 6 hr following treatment with cisplatin cytochrome *c* release was increased. A 3-hr treatment with 5 μM cisplatin

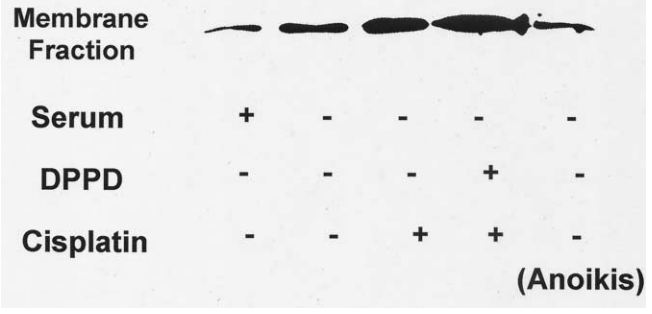

Fig. 10. Effect of DPPD and forced suspension on Bax translocation. M-1 cells were treated with 5 μM cisplatin in the absence and presence of 10 μM DPPD, and cytosolic and membrane fractions were isolated from control cells. Protein samples were also obtained from cells that were cultured in a petri dish for 6 hr to prevent their attachment. The levels of Bax in the cytosolic and mitochondrial membrane fractions were analyzed by immunoblot analysis as described under "Materials and methods.

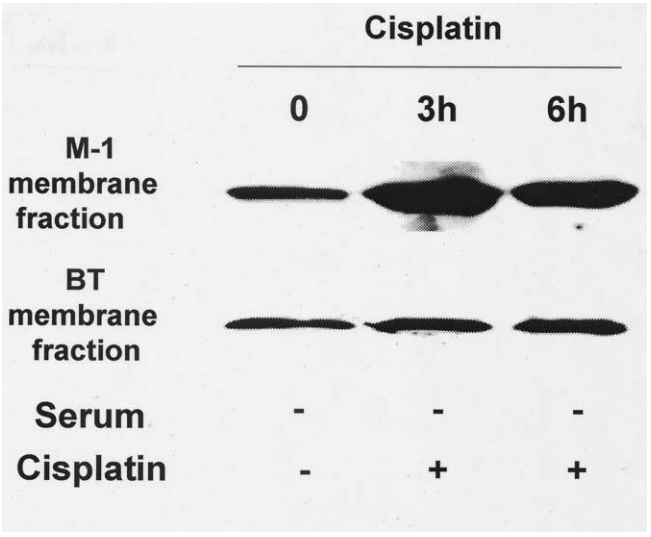

Fig. 11. Effect of Bcl-2 overexpression on Bax translocation. Cytosolic and membrane fractions were isolated from control cells or cells grown in serum-free medium for 3 or 6 hr in the absence and presence of 5 μM cisplatin. The levels of Bax in cytosolic and mitochondrial membrane fractions were analyzed by immunoblot analysis (as described under "Materials and methods").

did not induce cytochrome *c* release (data not shown). In contrast, a 2-hr treatment with a high concentration of cisplatin (0.5 mM) induced cytochrome *c* release, which was blocked by treatment with 10 μM DPPD.

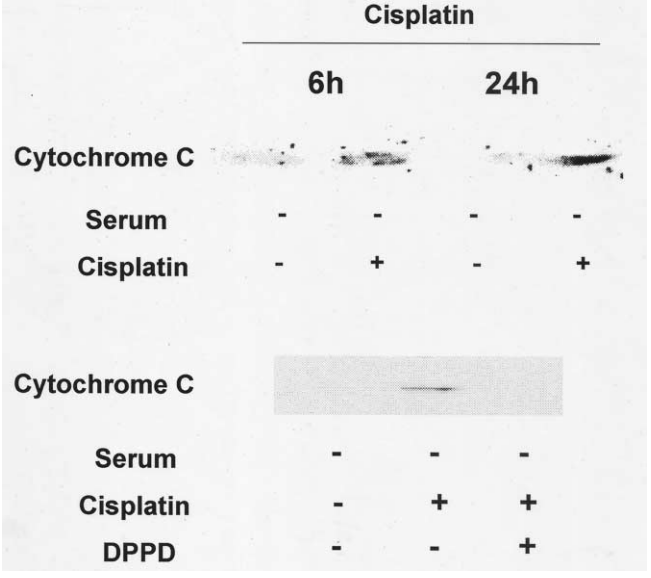

Fig. 12. Effect of cisplatin on cytochrome *c* release. Cytosolic fractions were isolated from control cells or cells grown in serum-free medium for 6 or 24 hr in the absence and presence of 5 μM cisplatin. To determine the effect of 0.5 mM cisplatin, cells were treated with cisplatin for 2 hr in the absence and presence of 10 μM DPPD. The levels of cytochrome *c* in the cytosolic fractions were analyzed by immunoblot.

4. Discussion

Our observation in this study that the mechanism of cell death induced by cisplatin was concentration dependent is consistent with the concept that the severity of cellular injury determines whether cells die by necrosis or apoptosis [14,19]. Apoptotic cell death occurs in response to a plethora of injurious stimuli, many of which can also induce necrosis [14,19]. High concentrations of a toxin or severe hypoxia typically result in necrosis, whereas lower concentrations of the same agents cause apoptosis [14,42]. In this study, treatment with a low concentration of cisplatin for 2 hr did not induce cell death, while treatment for 24 hr induced DNA fragmentation and significant cell death; a high concentration of cisplatin resulted in significant cell death after a 2-hr treatment, but did not induce overt DNA fragmentation after a 24-hr treatment (Figs. 1 and 2). These data indicated that high and low concentrations of cisplatin induced different modes of cell death. That is, cell death induced by high and low concentrations of cisplatin may be related mostly to necrosis and apoptosis, respectively. However, we cannot exclude the possibility that high concentrations of cisplatin can also induce apoptotic cell death, although the percentage of cases is low. In this study the cytocidal effects of cisplatin were found to be concentration dependent as was also observed previously by others in primary cultures of mouse proximal tubular cells [15].

The cytotoxic effect of cisplatin has been described to be related to the generation of ROS [21,22]. However, Kruidering *et al.* [23] reported that although ROS formation does occur during cisplatin-induced toxicity in porcine renal proximal tubular cells, it is not the direct cause of cell death. Lieberthal *et al.* [15] discovered that ROS play a role in mediating cisplatin-induced apoptosis but not necrosis in renal proximal tubule cells. In this study, DFO and DPPD provided substantial protection from necrotic cell death induced by high-concentration cisplatin, but neither DFO, BHA nor DMTU had an effect on DNA fragmentation and cell death induced by low concentrations of cisplatin (Figs. 3 and 4), which further supports the hypothesis that the cytocidal effects of cisplatin are concentration dependent. Thus, apoptosis in M-1 cells probably occurs by mechanisms that are independent of ROS.

The protective effect of an iron chelator on cisplatin-induced cell death in the present study has been taken as evidence for the participation of the hydroxyl radical in cell injury, because iron can promote hydroxyl radical production via the Fenton/Haber-Weiss reactions [43]. However, the hydroxyl radical scavenger DMTU and BHA did not attenuate the cisplatin-induced cell injury. DMTU also failed to protect cisplatin-induced LDH release in rabbit cortical slices [23]. Therefore, it is suggested that cisplatin induces necrotic cell death via iron-dependent mechanisms that are not associated with the formation of hydroxyl radicals in M-1 cells [44].

Recent reports indicated that the SAPK/JNK that phos-

phorylates the NH_2-terminal region of Jun [45] is involved in controlling apoptosis in certain systems. SAPK/JNK activation induces apoptosis in various cells including both neuron [39,46] and leukemia [47] cells. Cisplatin also activates SAPK/JNK in various cells [30]. In this study, SAPK/JNK activity was not altered by cisplatin treatment (Fig. 8), indicating that SAPK/JNK activity is not associated with cisplatin-induced apoptosis in M-1 cells. A lack of correlation between JNK activation and apoptosis has also been reported in other cells including B cells [48] and MDCK cells [49].

A recent study showed that cisplatin induces apoptosis in testicular carcinoma cells by activation of p53 [50]. However, M-1 cells were immortalized by overexpression of SV-40 antigen, which inhibits the function of p53 [51]. Therefore, the involvement of p53 in cisplatin-induced apoptosis can be excluded.

Different members of the Bcl-2 family either protect cells from cell death (for example, Bcl-2, Bcl-$X_{L,}$ and Bcl-w) or promote cell death (for example, Bcl-$X_{S,}$ Bad, Bak, Bax, and Bik), and undergo homo- and heterodimerization, depending upon their expression levels [52]. The balance of cell death protectors and inducers in the Bcl-2 family of proteins has been proposed as a common determinant of the susceptibility of cells to apoptotic cell death induced by various stimuli [52,53]. The present study showed that treatment with cisplatin for 24 hr in serum-free medium did not induce any significant changes in the protein levels of Bak, Bax, and Bcl-X_L (Figs. 5 and 6). Bcl-X_L and Bcl-2 mRNA levels analyzed in M-1 cells by RT–PCR were not changed after a 24-hr treatment with cisplatin. Therefore, changes in expression levels of the Bcl-2-related protein were not related to cisplatin-induced apoptosis. Similar findings have been observed in testicular germ cells [1]. However, we could not exclude the possibility that post-translational modification of Bcl-2 may be involved in the cisplatin-induced apoptotic process in M-1 cells. It has been reported recently that SAPK/JNK phosphorylates Bcl-2, which inhibits its action [54,55]. However, the possibility is not applicable to our system, because cisplatin did not affect SAPK/JNK activity in M-1 cells in this study.

As the levels of Bcl-2-related proteins do not change during apoptosis, it seems plausible that a post-translational activation of Bcl-2-related proteins occurs during apoptosis. Involvement of endogenous Bax translocation has been reported in apoptosis induced by hypoxia in renal epithelial cells [56] and by treatment with staurosporin or EGTA in neuroblastoma cells [57]. In this study, cisplatin induced Bax translocation to the membrane fraction (Fig. 9), indicating that translocation of endogenous Bax before nuclear fragmentation plays an important role in cisplatin-induced apoptosis, although other mechanisms such as Bcl-2 phosphorylation cannot be excluded completely. This notion was further supported by the data that Bcl-2 overexpression inhibited cisplatin-induced apoptosis and Bax translocation.

It is increasingly clear that mitochondria constitute an important component of the cell death machinery. Since mammalian cells are dependent upon mitochondrial function for long-term viability, irreversible mitochondrial damage is certain to assure a lethal outcome. Shimizu *et al.* [58] showed that Bax and Bak accelerate the opening of the voltage-dependent anion channels (VDAC) of mitochondria and allow cytochrome *c* to pass through them. Released cytochrome *c* activates caspase cascades, which are critical for the execution phase of apoptosis [59]. In this study, 0.5 μM and 0.5 mM cisplatin induced cytochrome *c* release, but the time course was different. That is, a low concentration of cisplatin induced cytochrome *c* release 6 hr after treatment, whereas a high concentration of cisplatin induced release at 2 hr. Cytochrome *c* release in necrotic cell injury has also been reported in cerebellar neurons, which may be mediated by ROS [60].

In this study, DPPD did not affect cisplatin-induced Bax translocation (Fig. 10) and a high concentration of cisplatin (0.5 mM) failed to induce Bax translocation to membrane fractions (Fig. 9), although treatment with DPPD protected cells against cisplatin-induced necrosis. This result indicates that Bax translocation does not play a significant role in acute cell injury by cisplatin.

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