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Inhibition of Apoptosis is Responsible for the Acquired Resistance of K562 Cells to Cisplatin

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Abstract – In an attempt to elucidate the role of apoptosis in drug resistance, cisplatin-resistant human chronic myelogenous leukemia (CML) K562 cells (K562/CDDP) were established and compared with drug sensitive parent cells (K562) in the induction of apoptosis. K562/CDDP cells were 5-fold more resistant to cisplatin compared to K562 cells. In addition, K562/CDDP cells were significantly more resistant to apoptosis as judged by DNA fragmentation and DAPI staining. K562/CDDP cells exhibited decreased proteolytic activity of caspase-3 and this was further demonstrated by decreased cleavage of its substrate poly (ADP-ribose) polymerase (PARP). Western blot analysis showed that K562/CDDP cells had longer sustained levels of BCL-X_L whereas no difference was noted in the level of Bcl-2. The translocation of Bax to mitochondria was significantly delayed in K562/CDDP cells. These results suggest that the reduced translocation of Bax and the sustained expression of Bcl-X_L may cause resistance to apoptosis through prevention of mitochondria release of cytochrome *c*, which subsequently induces reduction of caspase-3 activity and that this response is partly responsible for the acquired resistance to cisplatin in K562 cells.

Keywords □ cisplatin, apoptosis, resistance, K562 cell, Bax translocation

cis-Diamminedichloroplatinum(II) (cisplatin) exerts its anticancer activity by forming 1,2-intrastrand cisplatin-DNA cross-links. A serious limitation in cancer chemotherapy with cisplatin is the development of drug resistance. Furthermore, the dose escalation required to overcome even a small increase in resistance of a tumor to cisplatin can cause severe toxicities in patients. Therefore understanding the molecular basis of cisplatin resistance and exploitation of these mechanisms could improve the clinical effectiveness of this anticancer drug (Gonzalez *et al.*, 2001).

Classical multidrug resistance (MDR) is frequently associated with overexpression of P-glycoprotein, a member of the ATP binding cassette (ABC) family of transmembrane transport proteins capable of expelling certain cytotoxic drugs and maintains their nonlethal intracellular level (Kartner *et al.*, 1985; German, 1996). However, development of MDR is often observed in the absence of ABC protein overexpression (Liu *et al.*, 1999), suggesting that other factors can play a role in MDR (el-Deiry 1997). Multiple mechanisms have been implicated in the development of cisplatin resistance including reduced accumulation of the drug, increased levels of glutathione (GSH) (Joncourt *et al.*, 1998), enhanced expression of metallothionein (MT) (Okazaki *et al.*, 1998), enhanced DNA repair, increased levels of Bcl-2-related antiapoptotic genes, and alterations in signal transduction pathways involved in apoptosis (Timmer-Bosscha *et al.*, 1992; Chu, 1994; Perez, 1998). But the molecular mechanism of cisplatin resistance remains largely unclear.

The induction of apoptosis in cancer cells is thought to be fundamental to the success of treatment for cancer. The Bcl-2 family members are intimately involved in the apoptosis (Reed, 1998), but the role of these proteins in drug-induced death is controversial. The members of the Bcl-2 family can be subdivided into anti-apoptotic proteins such as *Bcl-2*, *Bcl-X_L*, and *Bcl-W* and pro-apoptotic proteins such as *Bax*, *Bad*, *Bid*, and *Bim*. It has been proposed that anti- and pro-apoptotic *Bcl-2* proteins regulate cell death by binding to each other and forming heterodimers (Oltvai *et al.*, 1993). Drugs induce endogenous *bax* expression through p53-dependent transcription in some cancer cell lines, but not in others (Miyashita *et al.*, 1995; Huang *et al.*, 2000).

Recently, several investigators reported that altered expression of apoptosis regulating proteins and subsequent resistance

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to apoptosis are deeply related with cisplatin-induced drug resistance in various cells. In the present study, we established cisplatin resistant K562 cells and attempted to clarify the relationship of apoptosis with drug resistance in K562 cells. In addition, the expression patterns of the apoptosis-regulating proteins were analyzed to elucidate mechanism of cisplatin-induced resistance.

MATERIALS and METHODS

Cell cultures

The p53 negative human chronic myeloid leukemia cell line K562 was obtained from Korean Collection for Type Cultures (KCTC). Cells were grown in RPMI 1640 supplemented with 10% FCS (Gibco BRL, Rockville, MD USA) in a 37°C humidified incubator under 5% CO_2. The cisplatin resistant sublines (K562/CDDP) were established by maintaining K562 cells in the presence of cisplatin over 12 months. Briefly, during the first 1 month, cells were grown in the presence of cisplatin at increasing concentrations of 0.3 μM and then cultured with 3 μM cisplatin for the following 3 months. The surviving cells were further maintained in the presence of 10 μM cisplatin for 12 months. When K562/CDDP cells were grown in the absence of cisplatin for 30 passages, their resistant properties sustained.

Cytotoxicity assay

Cell viability was determined by employing the cell proliferation reagent WST-1 (Roche Diagnostics GmbH, Manheim Germany), a tetrazolium salt that is cleaved by mitochodrial dehydrogenase in viable cells. Briefly, 198 μl of cell suspension (containing 1×10^4 cells) was plated in each well of 96-well plates. Cells were cultured for overnight to allow stabilization and then treated with various concentrations of cisplatin. After 72 hr incubation, 20 μl of WST-1 was added to each well and incubation was carried out at 37°C for 3 hr. Measurement of optical density in 450 nm was done using an automated plate reader (Becton-Dickinson, San Jose, CA USA).

Cisplatin-induced DNA fragmentation

DNA was isolated as previously described (Walker *et al.*, 1999) with minor modification. Briefly, cells following treatment were resuspended in 0.5 ml phosphate buffered saline, followed by 0.5 ml 2× neutral lysing solution (0.2 M NaCl, 10 mM EDTA, 20 mM Tris-Cl, pH 8.0, and 1% Sodium dodecyl sulfate) and 50 μl of proteinase K solution (10 mg/ml). After an incubation for 10 hr at 60°C, the samples were extracted with phenol/chloroform, the DNA precipitated with 2 volumes of ethanol at -70°C, washed in ice-cold ethanol, rehydrated (10 mM Tris-Cl, 1 mM EDTA, pH 8.0), and treated with 8 units of RNase for 4 hr. Electrophoresis in 1.8 % agarose (FMC, Rockland, Maine USA) gels containing 50 μg/ml ethidium bromide using Tris borate buffer (pH 8) was carried out in a gel electrophoresis system at 50 voltage for 2 hr. The gel was photographed under UV light (Transilluminator, Ultra-Violet Products Inc., San Gabriel; Polaroid film 667).

DAPI staining

DAPI staining was performed as described previously (Kim *et al.*, 1997). In brief, prior to staining, the cells were fixed with 4% paraformaldeyde for 30 min at room temperature, and then washed with PBS. DAPI (Sigma, St. Louis, MO USA) was added to the fixed cells for 30 min, after which they were examined by fluorescence microscopy (Olympus BX50, Tokyo, Japan). Apoptotic cells were identified by condensation and fragmentation of nuclei. Percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cell counted ×100. A minimum of 300 cells was counted for each treatment.

Subcellular fraction

Cells were harvested into 1ml of subcellular fraction buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiotheritol, 10 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Cells were then mechanically lysed at 4°C with 50 strokes of a cell homogenizer. The cell lysate was then spun at 1,000 ×g for 10 min, and the supernatant was further spun at 13,000 ×g for an additional 20 min. This pellet was resuspended in subcelluar fraction buffer contained 1% Triton X-100 and termed the mitochondrial pellet. The supernatant was termed the cytosolic fraction.

Immunoblotting

For immunoblot analysis, cells were harvested in 1mL lysis buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 50 mM β-glycerol phosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Na_3VO_4, and 5 mM NaF). The resulting lysates were resolved on a 4-15% tricine gels (30 μg per lane) and transferred onto Immobilon-P transfer membranes (Milipore, Bedford, MA, USA). The membranes were blocked with TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1%

Tween 20) containing 5% skim milk and then hybridized with different antibodies. Proteins were detected by using Supersignal reagents (Pierce, Rockford, IL USA).

RESULTS

Development of K562/CDDP Cells and Resistance to Apoptosis

K562/CDDP cells, a cisplatin-resistant variant of human leukemia K562 cells, were established by maintaining the cells with step-wise increasing concentrations of cisplatin over 10 months. K562/CDDP was about 5-fold more resistant to cisplatin than parent K562 cells; IC_{50} values were 1.73 and 8.43 μg/ml for K562 and K562/CDDP cells, respectively. The cisplatin-resistant phenotype of K562/CDDP was stable and resistance property remained unchanged after 30 passages.

No significant differences in intracellular drug concentration or the expression of p-glycoprotein were observed between K562/CDDP and K562 cells (data not shown). These results rendered us to investigate whether apoptosis plays a role in the development of resistance in K562 cells. Cisplatin-induced apoptosis in K562/CDDP cells was compared with that in drug-sensitive parental K562 cells using DNA fragmentation assay, and DAPI staining. K562 cells responded to cisplatin with internucleosomal DNA fragmentation in a time- and dose-dependent manner whereas no detectable DNA fragmentation was seen following exposure of K562/CDDP cells up to 100 μM CDDP (Fig. 1a). The frequencies of apoptotic cells were also assessed after staining the cells with DAPI. The percentage of apoptotic cells in K562 cells was at least 2-fold higher than those in K562/CDDP cells over the concentration of 0.3 to 5 μg/ml cisplatin (Fig. 1b). Resistance to apoptosis was further confirmed by FACS analysis of the cells after propidium iodide staining (data not shown).

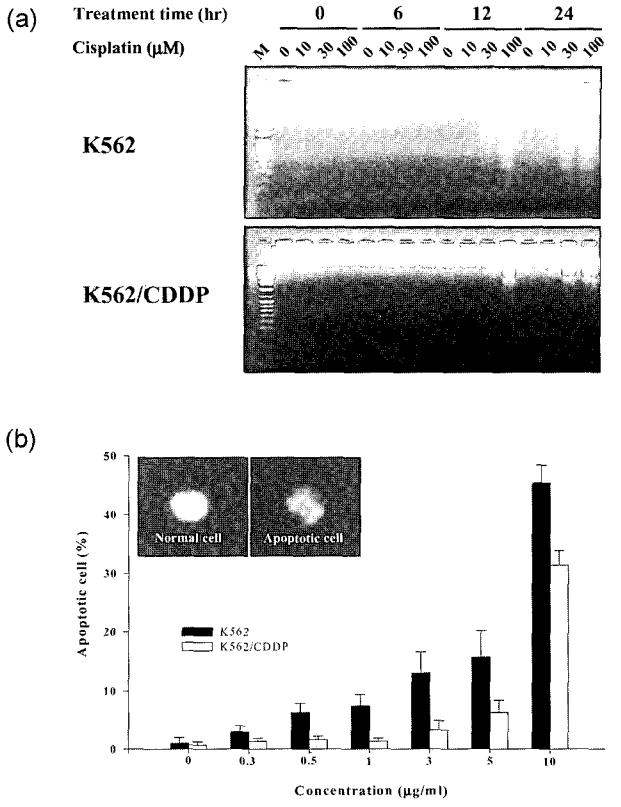

Fig. 1. Combined analysis of apoptosis by DNA gel electrophoresis and DAPI staining in K562 and K562/CDDP cells. (a) K562 and K562/CDDP cells were treated with different concentrations of cisplatin for the indicated times and then DNA was isolated. DNA fragmentation analysis was performed by electrophoresis in 1.8% agarose gels with 1 kb DNA ladder as size marker (M). (b) DAPI staining was used to determine the population of apoptotic cells in both K562 and K562/CDDP cells treated with various concentrations of cisplatin for 24 hr.

Decreased Caspase-3 Activation and PARP Cleavage in K562/CDDP Cells

Proteolytic activation of caspase-3 is one of the most important executioners in apoptosis. To test whether inhibition of apoptosis in K562/CDDP cells may be through reduced caspase-3 activity, 17 kD caspase-3 active forms from proteolytic cleavage were examined. Western blot analysis showed that levels of 17 kD caspase-3 in K562/CDDP cells were significantly reduced compared to those in parental K562 cells over the concentrations of 3-100 μM cisplatin (Fig. 2). Proteolytic activation of caspase-3 leads to its cleavage of multiple critical cellular proteins, including PARP. Therefore, PARP cleavage was examined in K562/CDDP cells in order to see whether reduced activation of caspase-3 may lead to reduced cleavage of PARP. Western blot analysis showed significantly lower level of PARP fragment in cisplatin-treated K562/CDDP cells than K562 cells (Fig. 2).

Decreased Levels of Mitochondrial Bax Protein in Cisplatin-Treated K562/CDDP Cells

Alteration of the expression of apoptosis regulating proteins was reported to frequently result in the dysregulation of apoptosis (Chao *et al.*, 1998; Reed, 2000). The expression of apoptosis-regulating proteins in K562/CDDP cells and K562 cells were analyzed after cells were treated with 0-30 μM cisplatin for 24 hr. There were no significant differences between K562/CDDP and K562 cells in the levels of anti-apoptotic protein

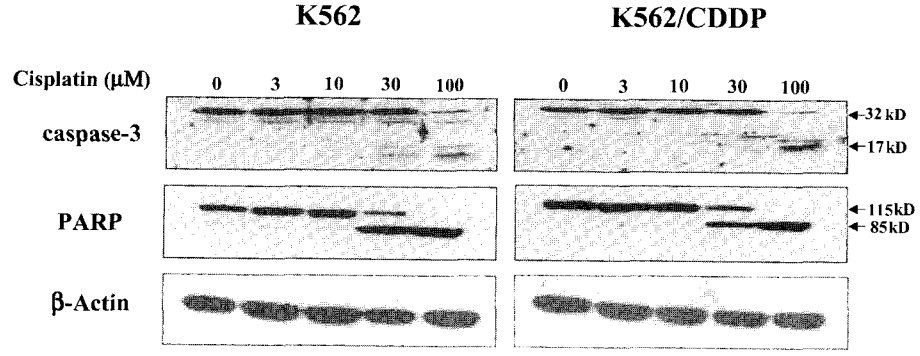

Fig. 2. Caspase-3 activation and PARP cleavage in K562 and K562/CDDP cells. Lysates from K562 or K562/CDDP cells treated with various concentrations of cisplatin for 24 hr were immunoblotted with mouse anti-caspase-3 and anti-PARP antibody. The position of caspase-3 active form and PARP cleaved form were denoted on the right of each panel by an arrow.

Bcl-2 either before or after cisplatin treatment. However, the expression of *Bcl-*X_L in K562/CDDP cells was more persisted than that in K562 cells (Fig. 3).

Subcellular distribution of *bax* is believed to be important in the induction of apoptosis. The level of *Bax* in cytosol of K562 cells was decreased after exposure to 30 μM cisplatin and completely disappeared 24 hr after the treatment. No significant changes in *Bax* protein levels in K562/CDDP cells occurred over the corresponding time course. *Bax* protein was not detected in mitochondrial membrane prior to cisplatin treatment in both cells, which is consistent with other reports (Finucane *et al.*, 1999; Nomura et al., 1999; Lee *et al.*, 2001). *Bax* protein appeared in mitochondrial membrane 9 hr after exposure to 30 μM cisplatin and the levels increased by 24 hr in K562 cells while it was detected only 18 hr after the exposure to the same concentration of cisplatin in K562/CDDP cells (Fig. 4).

Mitochondrial apoptotic function is then altered with the release of cytochrome *c*, which triggers a cascade of caspase activity resulting in cell death with the characteristic pattern of

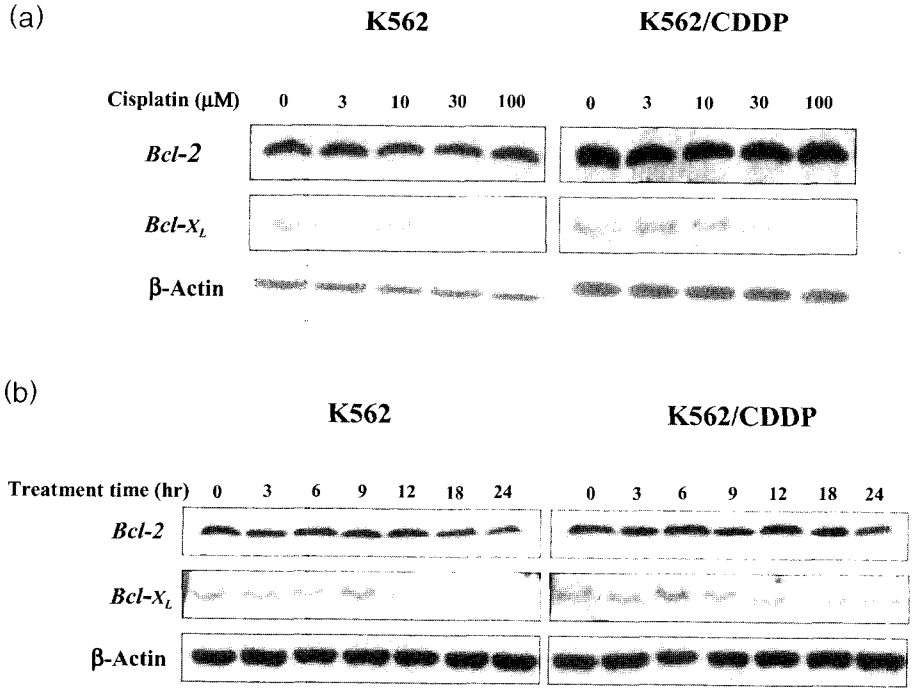

Fig. 3. Expression of *Bcl*-2 and *Bcl-*X_L in K562 and K562/CDDP cells. Lysates from K562 and K562/CDDP cells treated with different concentrations of CDDP (a) or treated with 30 μM cisplatin for the indicated times (b) were immunoblotted with anti-*Bcl*-2 and anti-*Bcl-*X_L antibody.

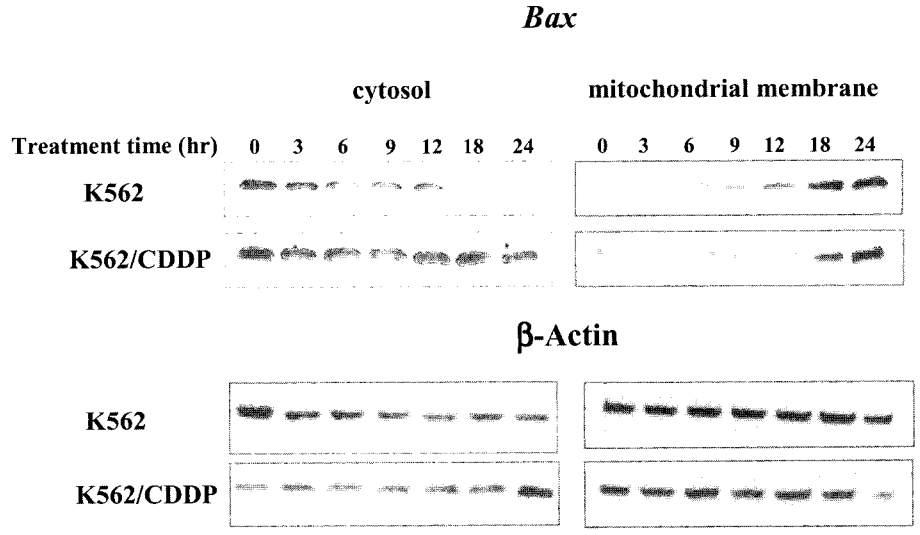

Fig. 4. Translocation of *Bax* protein from cytosol to mitochondrial membrane in K562 and K562/CDDP cells. K562 and K562/CDDP cells were treated with 30 μM cisplatin for the indicated times. After the treatment, cytosol and mitochondria were fractionated and immunoblotted with anti-*Bax* antibody.

cellular DNA degradation. Treatment of K562 cells with 30 μM cisplatin resulted in release of cytochrome *c* from mitochondria to cytosol. Cytochrome *c* appeared 6 hr after the treatment. On the contrary, the release of cytochrome *c* was significantly delayed in K562/CDDP cells (Fig. 5).

DISCUSSION

Resistance of tumor cells to chemotherapeutic agents is a major obstacle for successful cancer chemotherapy. Although multiple mechanisms have been implicated to be involved in chemoresistance, recent studies have demonstrated that chemically induced drug resistance of various cancer cells is deeply related with alterations in apoptosis (Kamarajan *et al.*, 2001; Li *et al.*, 2001; Liu *et al.*, 2002). Our preliminary results indicated that the P-glycoprotein was not involved in drug resistance in K562/CDDP cells as judged by intracellular drug concentration and expression level of p-glycoprotein (data not shown). It is important to keep in mind that the relationship between resistance to apoptosis and resistance killing remains to be proven. While it is possible that alterations in the apoptotic machinery contribute to clinical drug resistance, the data supporting this hypothesis remains scant.

The mitochondrial pathway play an important role in the regulation of apoptosis in mammalian cells because apoptotic signals induce mitochondria to release cytochrome *c*. Released cytosolic cytochrome c, along with apoptotic protease activating factor-1 (Apaf-1) and caspase-9, form a complex termed the "apoptosome" which facilitates caspase-9 activation, which then activates downstream caspase such as caspase-3. In addition, alteration of the expression of apoptosis regulating proteins was reported to frequently result in the dysregulation of apoptosis. These diverse response pathways converge on mitochondria, often through the activation of a pro-apoptotic mem-

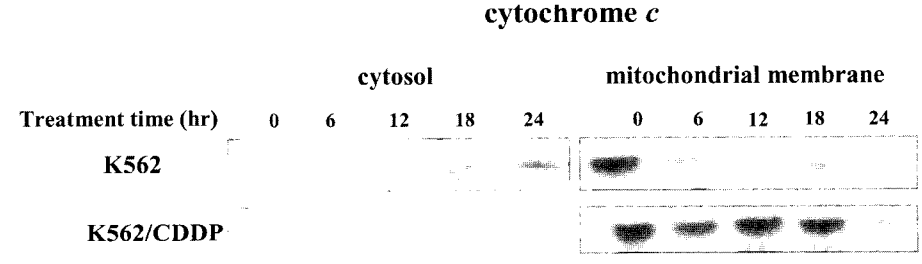

Fig. 5. Time-dependent distribution of cytochrome *c* in K562 and K562/CDDP cells. K562 and K562/CDDP cells were treated with 30 μM cisplatin for the indicated times. After the treatment, cytosol and mitochondrial membrane were fractionated and immunoblotted with anti-cytochrome *c* antibody.

ber of the Bcl-2 family (Harris *et al.*, 2000). Unlike *Bcl-2* that seems to spend most if not all of life attached to intracellular membranes, *Bax* can shuttle between the cytosol and organelles. The cytosolic forms represent pools of inactive, but battle-ready proteins. Pro-apoptotic signals redirect these proteins to the mitochondria, where the fight for the cells fate will take place. Activation of pro-apoptotic members can occur through proteolysis, dephosphorylation and probably several other mechanisms (Breitschopf *et al.*, 2000). *Bax*, a proapoptotic member of the Bcl-2 family, is a cytosolic protein that translocates to mitochondrial membrane upon induction of apoptosis, which subsequently release cytochrome *c*. Our immunoblot analysis showed a lower level of cytosolic *Bax* protein in cisplatin-treated K562 cells compared to K562/CDDP cells. *Bax* protein in mitochondrial membrane appeared in K562 cells occurred earlier than that in K562/CDDP cells. These results suggest that the reduced *Bax* translocation from the cytosol to the mitochondrial membrane be presumed to contribute to the resistance to cisplatin in K562/CDDP cells. The prevention of *Bax* translocation consequently blocks the release of cytochrome c and the activation of caspase-3.

The expression of antiapoptotic protein *Bcl-2* and *Bcl-X_L* was different between K562 and K562/CDDP cells. The levels of *Bcl-2* protein in K562/CDDP cells were similar to those in K562 cells whereas the expression of *Bcl-X_L* in K562/CDDP cells sustained higher than that in K562 cells. These results suggest that the constant expression of *Bcl-X_L* in K562/CDDP cells treated with cisplatin might prevent the translocation of *bax* to mitochondria and subsequently block the release of cytochrome c. *Bcl-X_L* expression can increase and sustain cell tolerance to DNA damage. *Bcl-X_L* may delay, or in some instances prevent, the activation of the death program, allow time for DNA repair. These events suggest that their antiapoptotic function may allow the cell to develop other avenues of drug resistance (Finucane *et al.*, 1999; Nagane *et al.*, 1998; Liu *et al.*, 1998). In addition, overexpression of *Bcl-X_L* prevented the translocation of *Bax* to mitochondria during apoptosis and this prevention blocked the release of cytochrome *c* (Ganju *et al.*, 2002).

In conclusion, our results demonstrated that cisplatin-induced acquired resistance is in part due to the inhibition of apoptosis in K562 cells. The resistance of K562/CDDP cells to apoptosis may be mediated by the prevention of *Bax* translocation and the sustenance of *Bcl-X_L* expression, which subsequently is responsible for the reduced release of cytochrome *c*.

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