Down-regulation of Bcl-2 is associated with cisplatin resistance in human small cell lung cancer H69 cells

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Abstract

Overexpression of the anti-apoptotic protein Bcl-2 has been associated with several malignancies, including small cell lung cancer (SCLC). In the present study, we have investigated if Bcl-2 contributes to the emergence of cisplatin resistance in SCLC H69 cells. The ability of cisplatin to induce apoptosis was decreased in H69 cells that acquired resistance to cisplatin (H69/CP). The level of Bcl-2 was, however, substantially reduced in H69/CP cells compared to parental H69 cells. There was little change in Bcl-2 content in H69 cells that were resistant to etoposide (VP-16) or Taxol. Bcl-2 was constitutively phosphorylated at serine 70 in H69 cells but not in H69/CP cells and cisplatin had little effect on Bcl-2 phosphorylation. The level of procaspase-3 was elevated in H69/CP cells but the ability of cisplatin to induce mitochondrial depolarization, caspase-9 activation, and poly(ADP-ribose) polymerase (PARP) cleavage was compromised in H69/CP cells. The level of the anti-apoptotic protein Bcl-xL and the pro-apoptotic protein Bak was slightly reduced in H69/CP cells but the ratio of pro-apoptotic and anti-apoptotic Bcl-2 family proteins was not sufficient to explain cellular resistance to cisplatin. These results suggest that the acquisition of cisplatin resistance by H69 cells was not due to an increase in the level/phosphorylation status of the anti-apoptotic protein Bcl-2. [Mol Cancer Ther. 2004;3(3):327–334]

Introduction

Lung cancer is the leading cause of cancer-related death in the United States and small cell lung cancer (SCLC) accounts for approximately 25% of all lung cancers. Unlike non-SCLC, which is intrinsically resistant to chemotherapy, 80–90% of SCLC patients initially respond to anticancer treatment although they are seldom curable by chemotherapy. The 2-year survival rate of SCLC patients is less than 10%. cis-Diamminedichloroplatinum(II) or cisplatin is frequently used for the treatment of SCLC (1). The majority of patients with SCLC, however, rapidly develop resistance to cisplatin causing therapy failure.

Most chemotherapeutic agents, including cisplatin, induce cell death by apoptosis. Activation of a family of cysteine proteases or caspases is essential for cell death by apoptosis (2). It is believed that DNA damage caused by chemotherapeutic drugs induces the release of mitochondrial cytochrome c, which facilitates activation of initiator caspase-9 thereby triggering activation of downstream effector caspases, such as caspase-3 (3). The activation of executioner caspases results in the cleavage of critical cellular proteins, such as poly(ADP-ribose) polymerase (PARP), DNA-dependent protein kinase, lamin B, and protein kinase Cδ (PKCδ). Apoptosis is regulated by a complex cellular signaling network and a defect in apoptotic signaling can contribute to drug resistance.

The proto-oncogene Bcl-2 discovered in low-grade Burkitt-cell lymphomas is a critical regulator of apoptosis (4). Overexpression of Bcl-2 has been associated with several malignancies, including SCLC (5, 6). Bcl-2 also plays an important role in cellular responses to chemotherapy. However, the involvement of Bcl-2 in SCLC has been controversial. While overexpression of Bcl-2 in SCLC increased resistance to drug-induced apoptosis (7), the survival of Bcl-2-negative tumors was less than Bcl-2-positive tumors (8).

There are at least fifteen members in the Bcl-2 family (4). While some members of the Bcl-2 family (e.g., Bcl-2 and Bcl-xL) suppress apoptosis, others (e.g., Bak, Bad, and Bax) enhance apoptosis (4). The pro- and anti-apoptotic family members can heterodimerize with each other and titrate each other’s function. The ratio between pro-apoptotic and anti-apoptotic Bcl-2 family members is an important determinant of cell survival and cell death (9).

Because cisplatin is the drug of choice for the treatment of SCLC and emergence of cisplatin resistance is a critical problem in cisplatin therapy, we examined if the anti-apoptotic protein Bcl-2 is associated with the acquisition of resistance by SCLC cells to cisplatin. Our results show that the constitutive level of Bcl-2 was high in SCLC H69 cells and Bcl-2 level was decreased considerably in cells that acquired resistance to cisplatin. In addition, Bcl-2 was phosphorylated in H69 but not in H69/CP cells. Furthermore, the ratio of anti-apoptotic to pro-apoptotic Bcl-2 family proteins was not sufficient to explain cisplatin resistance in H69/CP cells.
Materials and Methods

Materials
Monoclonal antibody to Bcl-2 and polyclonal antibodies to Bax, Bcl-xL, Bad, and β-tubulin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibodies to Bak and Bcl-2 were purchased from ImageGen Corporation (San Diego, CA). Monoclonal antibody to PARP and polyclonal antibodies to caspase-3 and caspase-9 were obtained from PharMingen (San Diego, CA). Phospho-Bcl-2 (Ser70) antibody was purchased from Cell Signaling Technology (Beverly, MA). JC-1 mitochondrial potential sensor was obtained from Molecular Probes (Eugene, OR). Horseradish peroxidase-conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from Jackson Immunoresearch Lab, Inc. (West Grove, PA). Cisplatin was purchased from Sigma (St. Louis, MO). Polyvinylidene difluoride membrane was from Millipore (Bedford, MA). Enhanced chemiluminescence detection kit and monoclonal antibody to actin were obtained from Amersham (Arlington Heights, IL).

Cell Culture
Parental small cell lung cancer H69 cells and cells selected for resistance to cisplatin (H69/CP0.4), etoposide (H69/VP-16), and Taxol (H69/Taxol) were generously provided by Dr. Nagahiro Saijo (National Cancer Center Research Institute, Tokyo, Japan). Cells were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 2 mM glutamine and 10% fetal bovine serum at 37°C in the presence of 5% CO2. H69/CP0.4 cells were further exposed to 1.0 μg/ml cisplatin to obtain H69/CP1.0 cells. Unless otherwise mentioned, H69/CP1.0 cells were used in all the studies. All the drug-resistant cells were maintained in drug-free media for at least 2 weeks before any experiment.

Reverse Transcription-PCR
Total RNA was extracted from H69 and H69/CP cells using RNA-STAT 60 reagent from Tel-Test, Inc. (Friendswood, TX). cDNA was synthesized using random primers and Improm II reverse transcriptase from Promega (Madison, WI). PCR amplification of cDNA was performed using thermal ace DNA polymerase (Invitrogen Carlsbad, CA), and Bcl-2 primers. The sequences of forward and reverse Bcl-2 primers were 5'-TATAAAGCTGTCGCA-GAGGGGCTA-3' and 5'-GTACTCAGTCATCCACAGGGC-GAT-3', respectively. After PCR cycling, a 480-bp product was produced. β-Actin was also amplified to generate an 800-bp product to use as a positive control.

Immunoblot Analysis
Cells were lysed in M-PER mammalian extraction buffer (Pierce, Rockford, IL) containing 1 mM DTT and protease inhibitors. Equal amounts of total protein were separated by 10% (w/v) SDS-PAGE and transferred onto a poly(vinylidene difluoride) membrane. Western blot analyses were performed as described before (10). The blot was probed with antibody to tubulin to control for equal loading.

Assessment of Apoptosis by Flow Cytometric Analysis
Cells were treated with and without cisplatin and incubated for various time periods. After incubation, cells were harvested and washed with PBS. Nuclei were isolated and stained with propidium iodide and DNA content was analyzed using a flow cytometer (Coulter Epics, Miami, FL) (11).

Assessment of Mitochondrial Membrane Potential by Flow Cytometric Analysis
Cells were incubated with 5 μg/ml of JC-1 for 30 min at 37°C, washed, and fluorescence was measured using a flow cytometer (Coulter Epics). JC-1 exhibits a potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (525 nm) to red (590 nm).

Results
Comparison of Bcl-2 Content in Parental and Drug-Resistant H69 Cells
Because overexpression of Bcl-2 has been associated with drug resistance, we examined if the level of Bcl-2 was elevated in H69 cells that acquired resistance to cisplatin. Figure 1 shows that the level of Bcl-2 was high in parental H69 cells but it was reduced considerably in cisplatin-resistant H69 (H69/CP) cells as compared to H69 cells. We also examined if Bcl-2 content was affected when H69 cells were selected for resistance to VP-16 or Taxol. As shown in Fig. 1, there was little change in Bcl-2 content in H69/VP-16 or H69/Taxol cells as compared to H69 cells. A modest decrease in Bcl-2 in H69/VP-16 cells could partly be due to loading differences as judged by the level of tubulin. Thus, the level of Bcl-2 was selectively reduced in H69 cells that acquired resistance to cisplatin.

To determine whether or not the decrease in Bcl-2 was at the transcriptional or posttranscriptional level, we performed reverse transcription (RT)-PCR using mRNA extracted from H69 and H69/CP cells. Figure 2 shows that the expression of Bcl-2 at the mRNA level was equivalent in H69 and H69/CP cells. Actin was used as a positive control.

Figure 1. Comparison of Bcl-2 content in parental and cisplatin-resistant H69 cells. Western blot analysis was performed with total cellular proteins using monoclonal antibody to Bcl-2. Tubulin was used to control for equal loading. Results are representative of two independent experiments.
Posttranslational modification of Bcl-2 can influence its anti-apoptotic function and phosphorylation of Bcl-2 at serine 70 plays a critical role in influencing anticancer drug sensitivity (12, 13). Therefore, we compared the phosphorylation status of Bcl-2 in H69 and H69/CP cells using an antibody that specifically recognizes phosphorylation status of Bcl-2 at Ser70. H69/CP0.4 and H69/CP 1.0 cells were selected with 0.4 and 1.0 \( \mu \)g/ml cisplatin, respectively. In addition, we have used H69/CP0.4 (Rev) cells that partially lost cisplatin resistance perhaps during prolonged culturing of cells in drug-free media. On the basis of the colorimetric cell proliferation assay [MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay], the degree of cisplatin resistance of H69/CP0.4, H69/CP1.0, and H69/CP0.4 (Rev) cells was 5.2-, 6.0-, and 2-fold, respectively. Figure 3 shows that Bcl-2 was constitutively phosphorylated in the parental H69 cells but phospho-Bcl-2 was undetectable in cisplatin-resistant H69/CP0.4 and H69/CP1.0 cells. The level of Bcl-2 as well as its phosphorylation status was low in H69/CP0.4 (Rev) cells. The blot was probed with tubulin to control for loading differences. We also examined the effect of cisplatin on Bcl-2 phosphorylation in H69 and H69/CP cells (Fig. 4). Cisplatin had little effect on Bcl-2 phosphorylation in H69/CP cells and it appears to decrease phospho-Bcl-2 level slightly in H69 cells. Thus, cisplatin resistance was not associated with an increase in Bcl-2 phosphorylation.

**Figure 2.** Comparison of Bcl-2 mRNA expression in H69 and H69/CP cells using RT-PCR. Total RNA was extracted from H69 and H69/CP cells and cDNA was synthesized by reverse transcriptase reaction. PCR amplification of Bcl-2 and actin (positive control) was performed and PCR products were subjected to gel electrophoresis as described under Materials and Methods. Results are representative of three independent experiments.

Posttranslational modification of Bcl-2 can influence its anti-apoptotic function and phosphorylation of Bcl-2 at serine 70 plays a critical role in influencing anticancer drug sensitivity (12, 13). Therefore, we compared the phosphorylation status of Bcl-2 in H69 and H69/CP cells using an antibody that specifically recognizes phosphorylation status of Bcl-2 at Ser70. H69/CP0.4 and H69/CP1.0 cells were selected with 0.4 and 1.0 \( \mu \)g/ml cisplatin, respectively. In addition, we have used H69/CP0.4 (Rev) cells that partially lost cisplatin resistance perhaps during prolonged culturing of cells in drug-free media. On the basis of the colorimetric cell proliferation assay [MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay], the degree of cisplatin resistance of H69/CP0.4, H69/CP1.0, and H69/CP0.4 (Rev) cells was 5.2-, 6.0-, and 2-fold, respectively. Figure 3 shows that Bcl-2 was constitutively phosphorylated in the parental H69 cells but phospho-Bcl-2 was undetectable in cisplatin-resistant H69/CP0.4 and H69/CP1.0 cells. The level of Bcl-2 as well as its phosphorylation status was low in H69/CP0.4 (Rev) cells. The blot was probed with tubulin to control for loading differences. We also examined the effect of cisplatin on Bcl-2 phosphorylation in H69 and H69/CP cells (Fig. 4). Cisplatin had little effect on Bcl-2 phosphorylation in H69/CP cells and it appears to decrease phospho-Bcl-2 level slightly in H69 cells. Thus, cisplatin resistance was not associated with an increase in Bcl-2 phosphorylation.

Anticancer agents can induce cell death both by apoptosis and necrosis. To examine whether the ability of cisplatin to induce apoptosis was impaired in cisplatin-resistant H69 cells, we monitored the appearance of sub-G1 peak indicative of apoptosis in a flow cytometer. Figure 5 shows that the treatment of H69 cells with cisplatin caused a time-dependent increase in sub-G1 peak; 33% and 54% of total population of cells underwent apoptosis when treated with 25 \( \mu \)m cisplatin for 48 and 72 h, respectively. The ability of cisplatin to increase the appearance of sub-G1 peak was attenuated in H69/CP cells such that only 13% and 22% cells appeared in the sub-G1 phase when treated with cisplatin for 48 and 72 h, respectively. We also examined if the ability of cisplatin to induce apoptosis was affected when cells develop resistance to VP-16 or Taxol. Figure 5 shows that the effect of cisplatin on the appearance of sub-G1 peak in H69/Taxol cells was equivalent to parental H69 cells but it was slightly reduced in H69/VP-16 cells. Therefore, H69/Taxol and H69/VP-16 cells were not resistant to cisplatin-induced apoptosis.

We also examined if apoptosis could be blocked by inhibition of caspases. We pretreated cells with the polycaspase inhibitor z-VAD before treatment with cisplatin and then repeated treatment with z-VAD after 24 h because peptide caspase inhibitors are highly unstable inside cells. On the basis of flow cytometric analyses, the caspase inhibitor was less effective in inhibiting cell death in H69/CP cells as compared to H69 cells; for example, z-VAD
prevented cell death by approximately 75% and 50% in H69 and H69/CP cells, respectively. z-VAD also partially inhibited cell death in parental and cisplatin-resistant H69 cells when cell survival was determined using the MTS assay (data not shown), suggesting that cisplatin may induce cell death not only by apoptosis but also by other mechanisms, such as mitotic catastrophe or necrosis. We also examined the effect of the PKC inhibitor rottlerin, which was shown to prevent cisplatin-induced apoptosis (10, 14). Figure 6 shows that rottlerin partially inhibited population of cells in the sub-G1 peak in H69 cells but not in H69/CP cells. In fact, rottlerin alone increased cell death in H69/CP cells.

We also monitored the ability of cisplatin to induce apoptosis by the cleavage of PARP, a substrate for effector caspase-3 and -7 (15). Figure 7 shows that cisplatin induced processing of Mr 116,000 full-length PARP to an Mr 85,000 fragment. The abundance of the Mr 85,000 cleavage product increased substantially when cells were treated with cisplatin for 48 h and it was processed further when cells were treated with cisplatin for 72 h. In contrast, the ability of cisplatin to induce PARP cleavage was dramatically reduced in H69/CP cells but not in H69/VP-16 and H69/Taxol cells. Furthermore, while VP-16 and Taxol induced PARP cleavage in H69 cells, the ability of VP-16 and Taxol to induce PARP cleavage in H69/CP cells, respectively, was compromised (data not shown). Thus, the ability of cisplatin to induce apoptosis was impaired in cisplatin-resistant H69 cells.

Comparison of the Effect of Cisplatin on Caspase Activation and Mitochondrial Membrane Depolarization in H69 and H69/CP Cells

Because Bcl-2 is known to inhibit mitochondrial cell death pathway (16), we compared the effect of cisplatin on the activation of caspase-9, the apical caspase in the mitochondrial cell death pathway, in H69 and H69/CP cells. Figure 7 shows that cisplatin induced processing of Mr 48,000 procaspase-9 to Mr 37,000 and 35,000 processed forms in H69 cells but not in H69/CP cells. Activation of caspase-9 was accompanied by processing of the downstream procaspase-3. The level of procaspase-3 was higher in H69/CP cells compared to H69 cells. However, cisplatin-induced activation of caspase-3, as judged by its conversion from its inactive proform to active processed forms and by the cleavage of its substrate PARP, was attenuated in H69/CP cells. Cisplatin, however, triggered processing of caspase-9 and caspase-3 in H69 cells that were resistant to VP-16 or Taxol. These results are consistent with the DNA fragmentation analysis determined by flow cytometry (Fig. 5).

Because Bcl-2 is localized in the outer mitochondrial membrane and is known to maintain mitochondrial integrity, we examined the effect of cisplatin on the loss of mitochondrial membrane potential (MMP) in cisplatin-sensitive and -resistant H69 cells (Fig. 8A). We have used the membrane potential sensitive cationic dye JC-1 to monitor mitochondrial membrane depolarization. At low concentrations, the dye exists as green fluorescent monomers in cells with low MMP. Membrane potential-driven accumulation of the dye in cells with normal mitochondrial function results in formation of red/yellow J1 aggregates. Consequently, mitochondrial depolarization can be monitored by the decrease in red/green fluorescence intensity ratio. As shown in Fig. 8A, we could identify three distinct populations of cells (A, B, and C). Eighty-six percent of untreated H69 cells exhibited highly polarized normal MMP (A). Treatment of H69 cells with cisplatin resulted in a decrease of red/green fluorescence ratio, indicating mitochondrial membrane depolarization (Fig. 8B). The percentage of cells with low MMP increased from 8.6% in untreated H69 cells to 62.8% in cisplatin-treated H69 cells. The loss of mitochondrial membrane potential was also observed in H69/CP cells treated with cisplatin (Fig. 8C). However, the percentage of cells with low MMP was significantly lower compared to H69 cells treated with cisplatin. This result is consistent with the DNA fragmentation analysis determined by flow cytometry (Fig. 5).
in a concentration-dependent decrease in cells with high MMP (A) with a concomitant increase in cells with intermediate (B) and low MMP (C) as evident by the loss of red fluorescence with an increase in green fluorescence. In H69/CP cells, almost 30% of cells remained in the intermediate B state. Low concentrations of cisplatin (≤25 μM) had only a little effect on the MMP but higher concentrations of cisplatin (50 μM) resulted in a decrease in cells in the B population with an increase in cells in the C population. We performed a parallel experiment to determine cell death induced by cisplatin (Fig. 8B). The extent of apoptosis as determined by the analysis of DNA fragmentation using a flow cytometer (Fig. 8B) correlated with mitochondrial membrane depolarization (Fig. 8A).

Discussion

The ability of cisplatin to induce cell death not only depends on its ability to induce DNA damage but also on cellular responses to DNA damage. The ultimate effect may be available antibodies. There was little alteration in Bak in H69/CP cells although the level of Bax was slightly reduced in H69/CP cells (Fig. 9). However, the ratio of anti-apoptotic and pro-apoptotic Bcl-2 family proteins was not sufficient to explain cisplatin resistance in H69/CP cells.
determined by events downstream of the drug-DNA interaction and the mechanism(s) of resistance may involve an alteration in cells' ability to trigger these downstream events. Various anti-apoptotic and pro-apoptotic molecules can influence the outcome of cell death. We have examined if the ability of cisplatin to induce cell death in H69/CP cells was affected by the anti-apoptotic protein Bcl-2. The results of our present study demonstrate that a decrease rather than an increase in Bcl-2 was associated with cisplatin resistance by SCLC H69 cells.

The primary function of Bcl-2 is to inhibit apoptosis. It is generally believed that an increase in Bcl-2 confers resistance to chemotherapeutic agents and down-regulation of Bcl-2 enhances chemosensitivity (9). In fact, antisense oligonucleotides targeting Bcl-2 were shown to enhance apoptosis in SCLC cells and synergized with chemotherapeutic agents (17, 18). Therefore, a decrease in Bcl-2 in cisplatin-resistant H69/CP cells was an unexpected finding. This is, however, consistent with the previous report that primary SCLC patients with Bcl-2-positive tumors had a complete remission rate of 40% versus 27% complete remissions in patients with Bcl-2-negative tumors after initial chemotherapy (8). Furthermore, overexpression of Bcl-2 in SCLC SBC-3 cells conferred resistance to Adriamycin, camptothecin, and mitomycin C but did not induce resistance to cisplatin, etoposide, VP-16 or Taxol (7).

Overexpression of the anti-apoptotic protein Bcl-xL has also been associated with SCLC (5, 6). However, antisense oligonucleotides targeted to Bcl-xL induced apoptosis in lung adenocarcinoma but not in SCLC cells (19). Our results show that the Bcl-xL level was not increased in H69/CP cells and therefore it could not compensate for the decrease in Bcl-2 content in H69/CP cells. We also examined if a decrease in pro-apoptotic family member, such as Bax or Bak, was associated with cisplatin resistance. Although, a slight decrease in Bax was noted in H69/CP cells, it was not enough to increase the ratio of anti- and pro-apoptotic Bcl-2 family members to account for cisplatin resistance.

Bcl-2 is a substrate for caspase-3, which cleaves Bcl-2 at the amino acid 34 and inactivates its survival function (20). It is believed that the cleavage of the NH2-terminal domain exposes the BH3 domain of Bcl-2 and the cleavage product of Bcl-2 acts as a pro-apoptotic protein rather than an anti-apoptotic protein (20). Cisplatin has been shown to cleave Bcl-2 at Asp 34 (21) to generate a Mr 23,000 fragment. Because an increase in Bcl-2 may also increase the generation of the pro-apoptotic form of Bcl-2 following treatment with cisplatin, this may explain why SCLC cells with elevated Bcl-2 were more responsive to cisplatin therapy. However, using an antibody that recognizes the variable loop domain (amino acids 41–54) of Bcl-2 and that has been shown to recognize the Mr 23,000 cleaved form of Bcl-2 (21), we were unable to detect any cleavage fragment of Bcl-2 in H69/CP cells or in H69 cells following treatment with cisplatin.

It is not clear why the level of Bcl-2 decreased in H69/CP cells. We have shown that the expression of Bcl-2 mRNA remained unaltered in H69/CP cells. It has been reported that PKC stimulation can increase the half-life of Bcl-2 mRNA (22). We have previously shown that the PKC signal transduction pathway was affected in cisplatin-resistant H69 cells (23). A decrease in PKC activity was associated with cisplatin resistance and this may contribute to destabilization of Bcl-2 message.

The function of Bcl-2 is also regulated by posttranslational modification although there are controversies whether Bcl-2 phosphorylation inactivates Bcl-2 or is required for its anti-apoptotic function (12, 13, 24). Phosphorylation of Bcl-2 at Ser70 has been shown to be an important regulator of its function (12, 13). Furthermore, PKCα has been recognized as a Bcl-2 kinase that phosphorylates Bcl-2 at Ser70 (24). We have shown that Bcl-2 is constitutively phosphorylated at Ser70 in H69 cells but not in H69/CP cells. This is consistent with our previous finding that PKCα level was reduced in H69/CP cells (23). We have found that cisplatin had little effect on Bcl-2 phosphorylation. In contrast, Taxol caused a marked increase in Bcl-2 phosphorylation in H69 cells but not in H69/Taxol cells (data not shown). This is consistent with the previous report that Bcl-2 phosphorylation is triggered by microtubule-targeting antineoplastic drugs (13). Several studies have reported that loss of Bcl-2 phosphorylation may confer resistance to apoptosis (25–27). Thus, a decrease in constitutively phosphorylated Bcl-2 may in fact contribute to resistance to cisplatin-induced apoptosis in H69/CP cells.
Bcl-2 can inhibit apoptosis either by directly inhibiting caspase activity or indirectly by controlling mitochondrial integrity (16). We have found that in H69/CP cells, a population of cells exists in a partially depolarized state. It has been reported that cells can remain viable after disruption of the outer mitochondrial membrane (28). While cisplatin caused mitochondrial membrane depolarization in H69 cells, the ability of cisplatin to decrease MMP was impaired in cisplatin-resistant H69 cells. Because apoptosis may proceed normally in cells in which mitochondria have been uncoupled (28), we also examined the effect of cisplatin on caspase-9 activation. The ability of cisplatin to cause activation of caspase-9 was also compromised in H69/CP cells as compared to H69 cells. We were unable to detect any caspase-8 in H69 cells but the level of procaspase-3 was elevated in H69/CP cells compared to H69 cells.

On the basis of both colorimetric cell proliferation assay and PARP cleavage, H69/CP cells were resistant to cisplatin compared to parental cells. In the present study, we have assessed cisplatin-induced apoptosis by flow cytometry and PARP cleavage. However, the caspase inhibitor z-VAD did not completely inhibit cisplatin-induced apoptosis in H69/CP cells. Although z-VAD is used as a poly caspase inhibitor, it may not inhibit all caspases. Furthermore, anticancer drugs may induce cell death not only by apoptosis but also by necrosis, oncosis, or mitotic catastrophe. Therefore, when caspase is inhibited, cells may die by these alternate mechanisms. Although the appearance of hypodiploid peak and PARP cleavage are used as criteria of apoptosis, it is conceivable that other modes of cell death may also contribute to DNA fragmentation and PARP cleavage.

The mechanism(s) of cisplatin resistance is often multifactorial (29). It has been demonstrated that cisplatin resistance in H69/CP cells cannot be explained by an alteration in drug accumulation, drug uptake or efflux, drug detoxification, or repair of interstrand DNA cross-link but was associated with an increase in cellular metallothionein, which is induced in response to a variety of cellular stress (30). We have previously shown that the PKC signal transduction pathway was affected in cisplatin-resistant H69 cells (23). The observation that PKCδ inhibitor rottlerin inhibited cisplatin-induced apoptosis in H69 cells but not in cisplatin-resistant H69/CP cells also supports the notion that an aberration in the PKCδ signaling may contribute to cisplatin resistance. It remains to be seen whether other cytoprotective pathways, such as activation of phosphatidylinositol-3 kinase/Akt, mitogen-activated protein (MAP) kinase, NF-κB, or XIAP are altered in cisplatin-resistant H69 cells.

Bcl-2 is widely used as a target for cancer chemotherapeutics. Antisense or small molecule inhibitors of Bcl-2 are being developed to down-regulate Bcl-2 to enhance anticancer drug sensitivity or to reverse drug resistance (17, 18, 31, 32). Our results suggest that over-expression of Bcl-2 may not contribute to acquisition of cisplatin resistance by SCLC H69 cells. However, a decrease in constitutively phosphorylated Bcl-2 in H69/CP cells appears to be associated with cisplatin resistance.

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