Cisplatin-induced Apoptosis Proceeds by Caspase-3-dependent and -independent Pathways in Cisplatin-resistant and -sensitive Human Ovarian Cancer Cell Lines

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ABSTRACT

We have assessed in detail the effect of cisplatin-activated programmed cell death in the cisplatin-sensitive human ovarian cancer cell line A2780 and two drug-resistant subclones, CP70 and C30. To determine whether the differential extent of apoptosis observed between the sensitive and resistant ovarian cancer cell lines was the result of dissimilar upstream signaling events, we assessed the execution of apoptotic events that preclude target protein proteolysis and subsequent chromosomal DNA degradation. Proteolytic degradation of procaspase-3 was observed in both the CP70 and C30 cells following IC50 cisplatin treatment, whereas no proteolyzed caspase-3 subunits were detected in the A2780 cells. However, using a direct enzymatic assay measuring cleavage of the synthetic peptide substrate (N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide), activity was detected in extracts prepared from A2780 cells treated at the IC50 level of cisplatin and was 2–3-fold less than that of extracts prepared from CP70 and C30 cells. Because the activation of procaspase-3 by caspase-9 requires the release of cytochrome c into the cytoplasm, we determined the level of cytosolic cytochrome c in each cell line in response to cisplatin treatment. Consistent with the caspase-3 activation data, a very small increase in cytosolic cytochrome c was observed in A2780 cells following cisplatin treatment, whereas dramatic increases were evident in both the CP70 and C30 cell lines. The expression of the mitochondrial factors Bcl-2, Bcl-x, and Bax was determined because each has been implicated in the regulation or release of cytochrome c at the level of the mitochondria. Bcl-2 and Bcl-xL proteins remained relatively unchanged in expression for over 48 h after exposure to cisplatin in the A2780 cell lines. However, within the same time period, expression of Bcl-2 decreased in the CP70- and C30-resistant cell lines, whereas an increase in Bcl-xL expression was observed. Expression of the proapoptotic Bcl-xL protein was observed in only the resistant CP70 and C30 cell lines independent of cisplatin treatment. A change in the expression of M, 24,000 Bax to a M, 21,000 isoform was evident in the A2780 cells within 48 h of cisplatin treatment and, to a greater extent, in the CP70 and C30 cells, which also expressed a M, 16,000 Bax variant. Evidence for an alternative apoptotic pathway in A2780 cells was obtained by demonstrating increased FADD expression in response to cisplatin treatment. These results support a model in which cisplatin-induced programmed cell death in the cisplatin-sensitive A2780 and -resistant CP70 and C30 cells proceeds via caspase-3-independent and -dependent pathways, respectively.

INTRODUCTION

Treatment of human ovarian cancer with the DNA-damaging agent cisplatin is, initially, an effective means by which to arrest malignancy. However, following preliminary success in tumor regression, recurrence and resistance to further chemotherapeutic treatment often ensues. This amplification in drug resistance has also been correlated to the development of a broad cross-resistance in ovarian cancer to other unrelated chemotherapeutic compounds. Factors affecting the occurrence of cisplatin resistance include: increased drug efflux, decreased drug influx, increased cellular glutathione levels, increased DNA repair, and drug tolerance (1–3).

Cisplatin is able to exert its cytotoxicity to ovarian cancer via the formation of mono-, inter-, and intrastrand cisplatin-DNA adducts, which can ultimately result in cell cycle arrests at G1, S, or G2/M and the induction of genetically PCD (4–7). PCD (3) proceeds in part due to aggregation and multimerization of upstream death effector molecules that concurrently or sequentially activate the cysteinyI aspartate-specific protease (caspase) cascade (8). Activated caspases ultimately target cytoplasmic and nuclear factors that maintain cellular architecture and are involved in DNA repair, replication, and transcription (8).

We have previously determined the degree of cisplatin-induced apoptosis in the human ovarian tumor cell lines A2780, CP70, and C30 (9). Both sensitive A2780 and resistant CP70 and C30 cell lines exhibited signs of apoptosis to some extent, contrary to an earlier report, which correlated acquired resistance to cisplatin in leukemic cells with a defect in the ability to activate the apoptotic process (10). Cleavage of two nuclear proteins, DNA-PKcs and PARP, was observed in the cell extracts prepared from all three cell lines but was ~2-fold more pronounced in the drug-resistant CP70 and C30 cell lines, compared to the drug-sensitive A2780 cell line. Cleavage of DNA-PKcs resulted in a decrease in the DNA-dependent kinase activity of the cells concurrent with a decrease in the DNA-binding activity of the Ku heterodimer component of DNA-PK. The production of nucleosomal-sized 180-bp DNA fragments was also observed in response to cisplatin-induced cell death, with the most dramatic effect visible in genomic DNA prepared from the two resistant cell lines. These data led to the hypothesis that there is a differential processing of apoptosis between the sensitive A2780 and resistant CP70 and C30 cell lines in response to cisplatin.

Here, we have correlated initial differences in the formation of apoptotic products in the A2780, CP70, and C30 human cell lines to differential expression of various pro- and antiapoptotic proteins among the sensitive and resistant cell lines. These results have allowed us to elucidate components involved in apoptosis in the A2780, CP70, and C30 cell lines following initiation of cisplatin-induced genetically PCD. Understanding the pathways by which cisplatin induces cell death and how these pathways are altered in resistant ovarian cancer can provide information necessary to target specific cell death pathways in the treatment of clinically resistant ovarian cancer.

MATERIALS AND METHODS

Materials. Cisplatin was from Sigma Chemical Co. (St. Louis, MO). The ovarian cancer cell lines A2780, CP70, and C30 were kindly supplied by Dr. T. C. Hamilton (Fox Chase Cancer Center, Philadelphia, PA). Cell culture
Cell Culture and Preparation of Cell-free Extracts. The A2780, CP70, and C30 human ovarian carcinoma cell lines were maintained in monolayer cultures, as we have described previously (9). Twenty-four h prior to cisplatin treatment, 3 x 10^6 cells from each cell line were plated into 150-mm tissue culture plates. To induce apoptosis, we treated cells for 4 h at 37°C at either equitoxic levels of cisplatin or with equivalent concentrations of drug. For equitoxic cisplatin experiments, A2780, CP70, and C30 cells were either untreated or treated with cisplatin at the corresponding IC_50 (1.92, 18.00, and 56.77 μM cisplatin, respectively) and IC_90 (7.95, 80.08, and 210.76 μM cisplatin, respectively) concentrations, as determined in a previous publication using clonogenic analyses to generate cisplatin cytotoxicity data (9). Live adherent cells, nonadherent cells, or a combination of both were collected at various time points after drug treatment, which did not exceed 72 h post-drug treatment. Whole cell extracts were prepared from cells, as described previously (9, 12). Protein concentrations of extracts were obtained using the microassay protocol of the Bio-Rad Assay Reagent with BSA as standards. All cell-free extracts were frozen at –70°C.

RESULTS

CASPASE-3 MEDIATES CISPLATIN-INDUCED APOPTOSIS

Fig. 1. Expression of caspase-3 in cisplatin-sensitive and -resistant human ovarian cancer cell lines prior to and following cisplatin treatment. Twenty μg of protein from each cell-free extract were separated by SDS-PAGE and transferred to Immobilon-P membranes, as described in “Materials and Methods.” Western blots were probed with a mouse monoclonal IgG anti-CPP32 antibody. Antibody reactivity was detected using Renaissance chemiluminescence reagents after a 1:3000 dilution of the secondary HRP-conjugated antibody. Lanes 1, 4, and 7, untreated; IC_50 (adherent) and IC_90 cisplatin-treated (nonadherent) A2780 cells, respectively; Lanes 2, 5, and 8, untreated, IC_50, and IC_90 cisplatin-treated (adherent) and (nonadherent) CP70 cells, respectively; and Lanes 3, 6, and 9, untreated, IC_50, and IC_90 cisplatin-treated (nonadherent) C30 cells, respectively. The positions of relative molecular weight standards are represented on the left, whereas the positions of the full-length caspase-3 molecule (p32) and its proteolyzed fragments (p20 and p17, respectively) are denoted on the right by arrows.

ac-DEVD-pNA Enzymatic Cleavage Activity. Cell-free extracts prepared 48 and 72 h following cisplatin treatment (20 μg) were incubated in 100 mM HEPES (pH 7.5), 20% glycerol, 0.5 mM EDTA, 0.1% BSA, 5 mM DTT, and 50 μM ac-DEVD-pNA colorimetric substrate (in 10% DMSO) for 1 h at 37°C. For assays using the caspase-3 inhibitor, z-DEVD-fmk, we initially incubated cell-free extracts with the inhibitor (50 μM in 10% DMSO) for 30 min at 37°C prior to addition of the colorimetric substrate. Production of cleaved p-nitroanilide from the tetrapeptide substrate ac-DEVD-pNA was monitored on a Molecular Dynamics SpectraMax 250 microplate reader at an absorbance of 405 nm. Assays were performed in triplicate, and the results are presented as the average increase in absorbance at 405 nm per min ± SD.

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active caspase-3 (17–19). The $M_c$ 12,000 small subunit of caspase-3 was not detected in these extracts because the peptide sequence used to generate the monoclonal antibody preceded the cleavage site that yields formation of the small subunit. Similar results in terms of the extent of caspase-3 cleavage were also obtained when each cell line was treated with 50 μM cisplatin for 48 h (data not shown). Proteolytic activation of caspase-3 remained undetectable in A2780, despite treatment with cisplatin at levels >5 times the IC$_{50}$. Because caspase-3 activation is an apoptotic event that likely precedes cell detachment and because the data presented above only assessed caspase-3 activation in the nonadherent cells following cisplatin treatment, we prepared extracts from cisplatin-treated cells encompassing both the adherent and nonadherent cells from the culture. Only the full-length procaspase-3 protein was detected in all three cell lines following IC$_{50}$ and IC$_{90}$ cisplatin treatment, demonstrating that the adherent cells do not contain activated caspase-3 at the levels observed in the nonadherent cells. The inability to detect activated caspase-3 in cell extracts prepared from both adherent and nonadherent cells is likely the result of the majority of the protein in these extracts being obtained from the adherent cells in the culture (data not shown).

**Determination of DEVD Cleavage Activity.** Our previous results demonstrated target protein cleavage of DNA-PKcs and PARP in A2780 cells following cisplatin treatment, albeit to a lesser extent than in CP70 and C30 cells. The inability to detect activated caspase-3 in A2780 cells following cisplatin treatment suggested that another caspase might be responsible for target protein cleavage in these cells. The synthetic tetrapeptide (ac-DEVD-pNA) substrate was used to detect protease activity because both DNA-PKcs and PARP are cleaved in A2780 cells following this sequence of amino acids. Release of p-nitroanilide from the peptide substrate in an *in vitro* spectrophotometric assay allows quantification of the amount of total DEVD-specific protease activity. Peptide cleavage activity was measured in extracts prepared from each cell line prior to and after cisplatin treatment, as well as following cisplatin treatment at both IC$_{50}$ and IC$_{90}$ levels. As expected, we detected no ac-DEVD-pNA cleavage activity in any of the cell lines prepared in the absence of cisplatin treatment (data not shown). Forty-eight (Fig. 2) and 72 h (data not shown) following treatment at the IC$_{50}$ and IC$_{90}$ doses of cisplatin (Fig. 2), ac-DEVD-pNA cleavage activity was observed in cell extracts prepared from nonadherent cells albeit significantly less in the A2780 cell line when compared to those from CP70 and C30 cells ($P < 0.004$ and $P < 0.001$). DEVD-specific peptide cleavage catalyzed by extracts prepared from nonadherent cells 48 h after cisplatin treatment at IC$_{50}$ levels of drug (Fig. 2, open bars) is ~65-fold more active in both the resistant cell lines when compared to the drug-sensitive counterpart. An increase in DEVD cleavage activity was observed in A2780 cells 48 h following treatment with cisplatin at the IC$_{50}$ levels. These data are consistent with our previous results demonstrating an increase in DNA laddering observed in A2780 cells following treatment with IC$_{90}$ levels of cisplatin (9). This activity could represent extremely low levels of activated caspase-3 that are below the detection limit of our immunoblot assay or the activation of a second caspase that is capable of cleavage following aspartic acid residues. No increase in DEVD cleavage activity was observed in the CP70 and C30 cell lines following treatment at IC$_{50}$ levels (Fig. 2, filled bars) compared to IC$_{50}$ levels of cisplatin (Fig. 2, open bars). This result was not unexpected because the activity values obtained for DEVD cleavage activity were normalized based on the mg of total protein, and the amounts of activity in detached cells following IC$_{50}$ and IC$_{90}$ treatment should be similar. The activity data in the CP70 and C30 cells are consistent with the activation of procaspase-3, suggesting that all of the DEVD cleavage activity is the result of caspase-3 in these cells. Similar results were obtained from cell extracts that were prepared 72 h after cisplatin exposure (data not shown). When extracts from cells treated at either IC$_{50}$ or IC$_{90}$ cisplatin were initially incubated with the irreversible, caspase-3-like inhibitor N-benzylxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone, no DEVD cleavage activity was detected prior to or following cisplatin treatment (data not shown). Cleavage of the DEVD substrate without the simultaneous detection of caspase-3 fragments, as observed in A2780 cells, suggests that another protease is responsible for target protein cleavage in A2780 cells following cisplatin treatment.

**Cytochrome c Expression.** The activation of the effector caspase-3 is catalyzed by the initiator caspase-9. Activation of procaspase-9 requires the release of cytochrome c into the cytoplasm, where a complex is formed with procaspase-9, Apaf-1, and dATP (20). The release of cytochrome c into the cytoplasm is thought to be the limiting factor in caspase-9 activation and subsequent activation of caspase-3 (20, 21). Therefore, we assessed the extent of cytochrome c translocation into the cytoplasm in A2780, CP70, and C30 cells in response to cisplatin treatment. The results shown in Fig. 3 demonstrate minimal levels of cytochrome c (M_c 14,000) in cytoplasmic extracts prepared from A2780, CP70, and C30 cells before drug treatment (Fig. 3, Lanes 1–3). Forty-eight h following cisplatin treatment at the IC$_{50}$ level, a dramatic increase in cytoplasmic cytochrome c was detected in both the CP70 and C30 cells (Fig. 3, Lanes 5 and 6). These data are consistent with the proteolytic activation of procaspase-3 (Fig. 1) and the activity determinations for DEVD cleavage (Fig. 2) in CP70 and C30 cells following cisplatin treatment. A small increase in cytoplasmic cytochrome c was also detected in A2780 cells following treatment (Fig. 3, Lane 4) and represents ~25% of that observed in the CP70 and C30 cell lines. Extracts were prepared from cells treated at the IC$_{90}$ level of cisplatin, similar results were obtained (data not shown). The increase in cytochrome c released into the cytoplasm was significantly greater in the CP70 and C30 cells compared to the A2780 cells, and no increase was observed comparing A2780 cells treated at IC$_{50}$ versus IC$_{90}$ levels of cisplatin. These data argue that the increased DEVD cleavage observed in A2780 cells is not the result of caspase-3 activation.

**Contribution of the Bcl-2 Family of Proteins in Cisplatin-induced PCD.** The release of cytochrome c from the intermembran-
the release of cytochrome c into the cytoplasm to initiate apoptosis (24). Therefore, we determined the level of expression of each of these mitochondrial components in the A2780, CP70, and C30 cell lines prior to and following cisplatin treatment.

Western blot and immunodetection analyses were performed using a mouse monoclonal IgG antibody that is specific for residues 41–54 of human Bcl-2, as described in “Materials and Methods.” The results shown in Fig. 4A demonstrate that Bcl-2 was minimally expressed and remained relatively unchanged in the A2780 cell line prior to and 48 h following treatment with cisplatin at the IC50 level (Lanes 1 and 4, respectively). These data are consistent with those previously reported by Jones et al. (27). Compared to untreated A2780 cells, the levels of Bcl-2 expression in untreated CP70 and C30 cells were 6- and 3-fold greater, respectively (Fig. 4A, Lanes 2 and 3). Following cisplatin treatment, the expression of Bcl-2 in CP70 and C30 cells reduced gradually over 48 h to near-background levels (Fig. 4A, Lanes 5 and 6; data not shown). Similar results for Bcl-2 expression were obtained in experiments treating cells at their respective IC50 cisplatin concentrations (data not shown). In vitro selection for acquired cisplatin resistance in A2780 cancer cells has been associated with the progressive development of cellular subpopulations that constitutively overexpress Bcl-2 (28, 29). Our data confirm the overexpression of Bcl-2 in resistant CP70 and C30 cells in the absence of treatment and show the level of Bcl-2 expression is lower in the resistant cells that have become committed to apoptosis. These data differ from those reported by Jones et al. (27), in which no change in Bcl-2 expression was detected in A2780 cells prior to and 48 h following treatment with cisplatin (Fig. 5A, Lanes 1 and 4). Following cisplatin exposure, a similar expression is observed (Fig. 5A, Lanes 4–6) in the detached CP70 and C30 cells.

These results suggest that Bcl-xS may counteract the effect of Bcl-xL and Bcl-2 to promote cisplatin-induced apoptosis in the CP70 and C30 cells. The lack of Bcl-xL expression in A2780 suggests a different mechanism must be used in these cells to induce PCD in response to cisplatin.

The induction of Bax has also been implicated in the initiation of chemotherapy-induced apoptosis (24, 26, 27, 33). Bax expression was...
The results thus far suggest that CP70 and C30 cells initiate apoptosis via a caspase-3-dependent pathway. However, even in the absence of caspase-3 activation, A2780 cells do exhibit some morphological characteristics of cisplatin-induced apoptosis, including a subset of the biochemical features of PCD. To investigate the possibility of an alternative apoptotic pathway in A2780 cells, we have assessed the expression of the Fas-associated death domain protein, FADD, which is activated in receptor-mediated apoptotic pathways (34). Interestingly, the basal level of FADD expression is greater in the A2780 cells compared to both the CP70 and C30 cells in the absence of cisplatin treatment (Fig. 6, Lanes 1–3). In addition, an increase in FADD expression was observed in the A2780 cells following treatment compared to untreated cells (Fig. 6, Lane 4). However, a decrease in FADD expression was observed in the CP70 and C30 cells following treatment compared to untreated controls (Fig. 6, Lanes 5 and 6, respectively). These results raise the interesting possibility that a separate apoptotic pathway may be used in A2780 cells in response to cisplatin treatment.

**DISCUSSION**

Using a tissue culture model for cisplatin resistant ovarian cancer, we have demonstrated cisplatin-induced apoptotic cell death in both the sensitive and resistant cell lines, as evidenced by target protein cleavage, genomic DNA degradation (9), and morphological assessments (data not shown). Closer examination of the apoptotic pathway in the A2780, CP70, and C30 cell lines has yielded interesting results with respect to the expression and activation of various components of the pathway which have been correlated with the ability to induce apoptosis in response to cisplatin. Resistance to cisplatin in ovarian cancer presents a continuous clinical problem. Our results suggest that cisplatin resistant ovarian cancer cells may use a different pathway to induce cell death compared to cisplatin-sensitive cells. This highlights an important difference with respect to the transduction of the cellular signal sensing DNA damage induced by cisplatin. By understanding the variety of pathways that contribute to cell death, one or more of...
these steps could be targeted to induce apoptosis in resistant ovarian cancer.

In response to cisplatin, the two resistant cell lines CP70 and C30 activated the classical DNA damage-induced apoptotic pathway (35). We observed target protein degradation (9), caspase-3 activation, and release of cytochrome c into the cytoplasm following cisplatin treatment. These late-stage apoptotic events were observed in conjunction with an alteration in the expression of some of the mitochondrial proteins known to be involved in the initiation of apoptosis. Expression of the antiapoptotic factor Bcl-2 was decreased following cisplatin treatment in resistant cells that were undergoing apoptosis, whereas expression was unchanged in the resistant cells that remained adherent 48 h following cisplatin treatment. Presumably, these cells represent those which are arrested in the cell cycle but have not yet initiated the apoptotic pathway. CP70 and C30 cells undergoing apoptosis also expressed higher levels of Bax protein following treatment, consistent with the proapoptotic activity attributed to Bax. The increase in Bax expression is also consistent with the increase in cytoplasmic cytochrome c observed in CP70 and C30 cells. Increased Bax expression following the induction of cell death has been correlated with the induction of the mitochondrial permeability transition and the mitochondrial release of cytochrome c (24, 36). Although we observed expression of the antiapoptotic factor Bcl-2, a protein that has been shown to block the Bax-dependent release of cytochrome c, the expression of Bax and Bcl-2 in the resistant cell lines likely abrogates the potential inhibitory activity of Bcl-2 following cisplatin treatment (Fig. 5; Ref. 31 and 37).

Our data showing Bax expression in CP70 cells in response to cisplatin treatment is consistent with a recent report demonstrating an increase in active and total p53 expression in these cells following cisplatin treatment (27) because Bax is known to be a target for transcriptional activation by p53 in response to DNA damage (33). Interestingly, in this report, no increase in Bax α expression was observed in CP70 cells following cisplatin treatment. The inability to detect Bax α was attributed to an alteration in the ratio of inactive to active p53, which increased following treatment. The p53 ratio was also higher when compared to A2780 cells following cisplatin treatment where the expression of Bax was detected. This result is consistent with ours, in that an increase in Bax α was observed in A2780 cells, albeit considerably less than that observed in either the CP70 or C30 cells following cisplatin treatment. It is important to note that different cisplatin treatment and cell extraction conditions were used; specifically, the level of cisplatin used did not induce apoptosis (27).

Recent results have demonstrated an increase in active p53 and transcriptional activation of the p21 gene in response to ionizing radiation. This effect was observed in both the A2780 cells and cisplatin-resistant cell line derived from the C30 cell line termed A2780/CP (38). However, no increase in active p53 was observed in response to cisplatin treatment. Again, it should be noted that cisplatin was used at a concentration of <20% of the IC50 (38). Our results clearly show an increase in Bax expression in CP70 and C30 cells that was accompanied by an increase in cytoplasmic cytochrome c and caspase-3 activation, consistent with numerous reports on the chemotherapeutic induction of the PCD pathway (35). A recent study using a transgenic mouse model for brain cancer has demonstrated that Bax acts as a tumor suppressor because Bax null mice exhibited an increase in cancer progression as a result of decreased apoptosis (39). This report placed Bax downstream of p53 but demonstrated that Bax was only responsible for 50% of the apoptosis. These results suggest that there is a Bax independent pathway for apoptosis as has been demonstrated in thymocytes in response to DNA damage (40). Although the status of p53 is important for apoptotic induction, clearly, there are tissue specific differences, and our results demonstrate differences associated with chemotherapeutics-induced resistance.

Although clear evidence for a caspase-3 dependent apoptotic pathway was obtained for CP70 and C30 cells, we were unable to detect activated caspase-3 in A2780 cells. However, we were able to detect DEVD-specific cleavage of a synthetic caspase-3 substrate and target protein degradation in the A2780 cells following cisplatin treatment (9). These results suggest that the caspase-3-dependent pathway is inactive in the A2780 cell line. Recently, caspase-3 has been specifically implicated as the effector caspase responsible for cleavage of the human DNA fragmentation factor (DFF) and the inhibitor of the murine caspase-activated DNase (ICAD; Refs. 14 and 15). Cleavage of DFF/ICAD then activates the DNA endonuclease (DFF–40/ICAD) required for formation of apoptotic DNA ladders (13, 16). Caspase-3 has also been shown to be necessary for the typical morphologies associated with apoptosis (41). The relatively inefficient formation of DNA ladders (9) and altered apoptotic morphology observed in A2780 cells (data not shown) is consistent with a lack of caspase-3 activation following cisplatin treatment. Therefore, other caspasess that are capable of cleaving an aspartic acid in the P1 position could be responsible for the relatively inefficient target protein degradation observed in the parental A2780 cells (9). Evidence for an alternative apoptotic pathway in A2780 cells was obtained by measuring an increase in expression of FADD, which participates in the Fas/Fas-L apoptotic pathway. Clearly, it will be of interest to determine the efficiency of FADD-mediated PCD in response to cisplatin treatment and the impact of this pathway on cellular resistance to cisplatin.

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