Inhibition of BAD Phosphorylation Either at Serine 112 via Extracellular Signal-regulated Protein Kinase Cascade or at Serine 136 via Akt Cascade Sensitizes Human Ovarian Cancer Cells to Cisplatin

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ABSTRACT

We studied the roles of the phosphatidylinositol 3-kinase (PI-3K)-protein kinase B/Akt-BAD cascade in both cisplatin-resistant Caov-3 and -sensitive A2780 human ovarian cancer cell lines. Treatment of both Caov-3 and A2780 cells with cisplatin but not with the trans-diaminodichloroplatinum (transplatin) isomer stimulated the activation of Akt, and the PI-3K inhibitor wortmannin blocked the cisplatin-induced activation of Akt. Treatment of both Caov-3 and A2780 cells with cisplatin but not with the trans-diaminodichloroplatinum isomer also stimulated the phosphorylation of BAD at both the Ser-112 and Ser-136 sites. Whereas the phosphorylation of BAD at Ser-136 was blocked by treatment with wortmannin, its phosphorylation at Ser-112 was blocked by a MAP/ERK kinase inhibitor, PD98059. Exogenous expression of a dominant-negative Akt in both Caov-3 and A2780 cells decreased the cell viability after treatment with cisplatin. In contrast, no sensitization to cisplatin was observed in cells expressing wild-type Akt. We further examined the role of BAD in the viability after cisplatin treatment using BAD mutants. Exogenous expression of each of the singly substituted BADS112A or BADS136A in both Caov-3 and A2780 cells decreased the viability after treatment with cisplatin to a degree intermediate between that caused by exogenous expression of wild-type BAD and doubly substituted BADS2A. Cisplatin did not stimulate the phosphorylation of BAD Ser-136, but did stimulate the phosphorylation of BAD Ser-112 in cells expressing a dominant-negative Akt, suggesting that BAD Ser-136 but not Ser-112 was phosphorylated by Akt. Our findings suggest that cisplatin-induced DNA damage causes the phosphorylation of both BAD Ser-112 via an extracellular signal-regulated protein kinase (ERK) cascade and BAD Ser-136 via a PI-3K-protein kinase B/Akt cascade and that inhibition of either of these cascades sensitizes ovarian cancer cells to cisplatin.

INTRODUCTION

The homeostasis of normal tissues is a balance between cell proliferation and death. Alterations of both pathways contribute to the clonal expansion of cancer cells. Apoptosis, or programmed cell death, is an active form of cell suicide. Recently, a signaling pathway by which extracellular stimuli suppress apoptosis has been characterized. One of the first reports on survival signaling linked activation of the Ras-MAPK signaling pathway by which extracellular stimuli suppress apoptosis has been characterized. One of the first reports on survival signaling linked activation of the Ras-MAPK signaling pathway by various cellular stimuli that control cell growth and differentiation cause a rapid increase in the enzymatic activity of a family of serine/threonine kinases known as the MAP kinase family. The MAP kinase family has been classified into three subfamilies: ERKs, including ERK1 and ERK2; stress-activated protein kinases, also termed JNKs, including JNK1 (46 kDa) and JNK2 (55 kDa); and p38 kinase, a homologue of the yeast HOG1 (high-osmolarity glycerol response-1) kinase (17). We recently reported that both ERK and JNK are activated by cisplatin-induced DNA damage and are required for cell survival after cisplatin treatment (18). The involvement of MEK upstream of BAD phosphorylation (19) and the promotion of cell survival by the Ras-MAPK signaling pathway by phosphorylation of BAD at Ser-112 (20–22) were reported recently. However, the effect of certain DNA-damaging agents on the PI-3K-Akt-BAD cascade remains to be elucidated. Therefore, we sought to determine whether the PI-3K-Akt-BAD cascade plays a role in the cellular stress response to the chemotherapeutic agent cisplatin, which damages DNA through the formation of bifunctional platinum adducts. For our study, we used both Caov-3 human ovarian cancer cells, which are resistant to cisplatin, and A2780 human ovarian cancer cells, which are sensitive to cisplatin. Here we provide evidence that cisplatin but not transplatin, which does not readily damage DNA (23, 24), induced the activation of Akt and phosphorylation of BAD. Moreover, inhibition of Akt and BAD markedly decreased cell viability after treatment with cisplatin.

MATERIALS AND METHODS

Materials. Wortmannin was purchased from Sigma Chemical Co. (St. Louis, MO). Geneticin was purchased from Life Technologies (Grand Island, NY). ECL Western blotting detection reagents were obtained from Amersham Pharmacia Biotech (Arlington Heights, IL). PD98059, rabbit polyclonal antiphospho-BAD (Ser-112) and BAD (Ser-136) antibody, rabbit polyclonal anti-Akt antibody, and the Akt kinase assay kit, which included GSK-3 fusion protein and a phospho-specific GSK-3β antibody, were obtained from New England Biolabs (Beverly, MA). Rabbit polyclonal anti-HA antibody was obtained from Santa Cruz Biotechnology.
Cell Cultures. Human ovarian papillary adenocarcinoma cell line Caov-3 was obtained from American Type Culture Collection (Rockville, MD). Human ovarian cancer cell line A2780, derived from a patient prior to treatment, was kindly provided by Dr. T. Tsuruo (Institute of Molecular and Cellular Biosciences, Tokyo, Japan) and Drs. R. F. Ozols and T. C. Hamilton (Fox Chase Cancer Center, Philadelphia, PA; Refs. 25, 26). The cells were cultured at 37°C in DMEM with 10% FBS in a water-saturated atmosphere of 95% O<sub>2</sub> and 5% CO<sub>2</sub>.

Constructs. The vector encoding the various HA-tagged forms of Akt, either wild-type or kinase-dead (K179M mutant), and the various forms of HA-tagged BAD, either wild-type (pcDNA3-BAD) or mutants (pcDNA3-BADS136A, pcDNA3-BADS112A, and pcDNA3-BADS S to A at 112 and 136) used in this study have been described previously (12).

Clone Selection. Caov-3 and A2780 cells were transfected for 12 h in 6-well tissue culture plates with 2 µg of the empty vector (CMV-6); with CMV-6 containing the gene for HA-tagged wild-type Akt or HA-tagged Akt K179M and the neomycin resistance gene; with the empty vector (pcDNA3), which contains a neomycin resistance gene; or with pcDNA3-BAD, pcDNA3-BADS136A, pcDNA3-BADS112A, or pcDNA3-BAD S to A at 112 and 136, using Lipofectamine plus (Life Technologies, Gaithersburg, MD; Ref. 18). Clonal selection was performed by adding geneticin to the medium at a final concentration of 200 µg/ml 2 days after the transfection. After 3 weeks, several clones were isolated using cloning rings. Selected clones were then maintained in medium supplemented with geneticin (100 µg/ml), and only low-passage cells (passage < 10) were used for the experiments described here. For analysis of the levels of ectopically expressed Akt and BAD protein products, empty vector (CMV-6), wild-type Akt, or Akt<sup>K179M</sup> expressing Caov-3 cells or empty vector (pcDNA3)-, wild-type BAD-, BADS112A-, BADS136A-, and BAD2SA-expressing Caov-3 cells cultured in 100-mm dishes were lysed in ice-cold HNTG buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 10 mM sodium PP<sub>i</sub>, 100 µM sodium orthovanadate, 100 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM phenethylsulfonyl fluoride (27)]. The lysate samples were immunoprecipitated with anti-HA antibody. Immunocomplexes were precipitated with protein A-Sepharose, and the isolated proteins were analyzed by electrophoresis on 8% SDS-polyacrylamide gels. Transfer to nitrocellulose, Western blotting with anti-Akt or anti-BAD antibody, and washing were performed as described elsewhere (27).

Cytotoxicity. Cell viability (18) was assessed by the addition for 1 h of cisplatin or transplatin 1 day after seeding 1.0 × 10<sup>5</sup> test cells into 96-well plates, followed by exchanging the medium with fresh medium. Because a significant level of DNA repair after cisplatin-induced DNA damage was detected 5 days later but not after 24–72 h (28), the number of surviving cells was determined using the Bio-Rad protein assay reagent. The samples were resuspended in 40 µl of kinase assay buffer containing 200 µM ATP and 1 µg GSK-3α fusion protein. The kinase reaction was allowed to proceed at 30°C for 30 min and stopped by the addition of Laemmlli SDS sample buffer (29). Reaction products were resolved by 15% SDS-PAGE followed by Western blotting with a phospho-GSK-3α antibody.

For analysis of the total amount of Akt, 250 µg of protein from the lystate samples were resolved by 8% SDS-PAGE, followed by Western blotting with anti-Akt antibody. For analysis of the effect of ectopically expressed Akt on Akt activity, empty vector (CMV-6)-, wild-type Akt-, or Akt<sup>K179M</sup> expressing Caov-3 cells grown in 100-mm dishes were treated with 1 mM cisplatin for 3 h. The lystate samples were immunoprecipitated with anti-HA antibody. Immunocomplexes were precipitated with protein A-Sepharose, and the kinase reaction was carried out in the presence of cold ATP and GSK-3α fusion protein, as described above.

Phosphorylation of BAD. Cells cultured in 100-mm dishes were transfected with 4 µg of pcDNA3-BAD, using Lipofectamine plus. At 72 h after transfection, serum-deprived cells were treated with various materials. They were then washed twice with PBS and lysed in ice-cold HNTG buffer (27). The lystate samples were immunoprecipitated with phospho-BAD (Ser-136) or phospho-BAD (Ser-112) antibody. Immunocomplexes were precipitated with protein A-Sepharose, and the isolated proteins were analyzed by electrophoresis on 8% SDS-PAGE. Transfer to nitrocellulose, Western blotting with phospho-BAD (Ser-112) or phospho-BAD (Ser-136) antibody, and washing were performed as described elsewhere (27).

For analysis of the total amount of BAD, 250 µg of protein from the lystate samples were resolved by 8% SDS-PAGE, followed by Western blotting with anti-BAD antibody. For analysis of the effect of ectopically expressed BAD on BAD phosphorylation, empty vector (pcDNA3)-, wild-type BAD-, BADS112A-, BADS136A-, and BAD2SA-expressing Caov-3 cells grown in 100-mm dishes were treated with 1 mM cisplatin for 3 h. The lystate samples were immunoprecipitated with anti-HA antibody. Immunocomplexes were precipitated with protein A-Sepharose, and the isolated proteins were resolved by 8% SDS-PAGE, followed by Western blotting with phospho-BAD (Ser-112) or phospho-BAD (Ser-136) antibody, and washing as described above (27).

Statistics. Statistical analysis was performed using Student's t test, and P < 0.01 was considered significant. Data are expressed as the mean ± SE.

RESULTS

Activation of Akt. To evaluate whether Akt is activated by cisplatin in Caov-3 or A2780 human ovarian cancer cells, cultured cells were exposed to 1 mM cisplatin for the indicated times (Fig. 1A) and at the indicated concentrations for 3 h (Fig. 1B). Cell lysates were immunoprecipitated with immobilized anti-Akt antibody, followed by addition of GSK-3α fusion protein and Western blotting with anti-phospho-GSK-3α/β antibody. Activation of Akt by cisplatin in Caov-3 cells was detected at 1 h, reached a plateau from 3 h through 6 h, and declined thereafter (Fig. 1A, top row). We confirmed that the total amount of Akt in each lane was the same (Fig. 1A, middle row). Activation of Akt by cisplatin in A2780 cells was also detected at 1 h, reached a plateau at 3 h, and declined thereafter (Fig. 1A, bottom row). Cisplatin induced the activation of Akt in a dose-dependent manner in Caov-3 (Fig. 1B) and A2780 cells (data not shown). It is known that cisplatin but not transplatin forms covalent cross-links between the N7 position of adjacent guanine or adenine-guanine residues (23, 24). Treatment with transplatin had no apparent effect on Akt activation, whereas cisplatin clearly induced Akt activation in Caov-3 (Fig. 1C, top row) and A2780 (Fig. 1C, bottom row) cells. Because Akt is an effector of survival signaling downstream from PI-3K, we next determined whether stimulation of both types of cells with cisplatin could increase the activity of Akt through a PI-3K-dependent mechanism. Both types of cells were stimulated with cisplatin in the presence or absence of wortmannin, a PI-3K inhibitor, and the kinase activity of Akt was assayed. The induction of Akt activity by cisplatin was inhibited by wortmannin (Fig. 1C, Lane 3). These results indicate that only the DNA-damaging cisplatin isomer activates Akt activity in both types of cells through a PI-3K-dependent mechanism.
Phosphorylation of BAD. In recent studies (12–14), BAD has been identified as a potential target of PKB/Akt, linking the PI-3K pathway directly to the apoptotic machinery. Therefore, we next examined the effect of cisplatin on the phosphorylation of BAD. BAD function is modulated by phosphorylation at two sites, Ser-112 and Ser-136 (14). Caov-3 (Fig. 2, top rows) or A2780 cells (Fig. 2, bottom rows) were transfected with pCDNA3-BAD and exposed to 1 mM cisplatin for 3 h. Cell lysates were immunoprecipitated with either anti-phospho-BAD (Ser-112; Fig. 2A) or anti-phospho-BAD (Ser-136; Fig. 2B) antibody, followed by Western blotting with the same antibody. Whereas transplatin had no effect on BAD phosphorylation (Fig. 2, Lane 4), cisplatin induced the phosphorylation of BAD at Ser-112 and Ser-136 in Caov-3 and A2780 cells (Fig. 2, Lane 2). Moreover, we confirmed that the total amount of BAD in each lane from a given cell line was the same (Fig. 2C). Because cisplatin-induced BAD phosphorylation, the relative amount of nonphosphorylated BAD was reduced by cisplatin, suggesting that cisplatin-induced BAD phosphorylation indicated the inhibition of the proapoptotic effect of BAD. In accordance with reports showing that Akt phosphorylates BAD specifically at Ser-136 (12, 13), cisplatin-induced phosphorylation of BAD at Ser-136 was inhibited by wortmannin (Fig. 2B, Lane 3). On the other hand, cisplatin-induced phosphorylation of BAD at Ser-112 was not inhibited by wortmannin (Fig. 2A, Lane 3). The presence of two phosphorylation sites on BAD suggests that the simultaneous activation of different survival pathways may result in the concomitant phosphorylation of BAD Ser-112 and Ser-136 by different kinase cascades (12). In addition, the possibility that MEK is involved upstream of BAD phosphorylation has been reported (19). Therefore, we examined the effect of PD98059, an MEK inhibitor, on BAD phosphorylation. Although pretreatment with PD98059 had no effect on the phosphorylation of BAD at Ser-136 (Fig. 2B, Lane 5), it attenuated the phosphorylation of BAD at Ser-112 (Fig. 2A, Lane 5).

Kinase-deficient Akt Sensitizes Caov-3 and A2780 Cells to Cisplatin but not Transplatin. To determine whether Akt activation is necessary for cell survival signaling after cisplatin-induced DNA damage, the effect of cisplatin treatment on the viability of Caov-3 (Fig. 3B) and A2780 cells (Fig. 3C) expressing a kinase-deficient Akt (AktK179M) was compared with that of an empty vector (CMV-6)-expressing control line. AktK179M cells express an Akt derivative rendered kinase-inactive by a point mutation within the catalytic domain (5, 30, 31). We first confirmed the overexpression of ectopically expressed Akt protein products (Fig. 3A, bottom row) and the negative effects of the expression of HA-AktK179M on Akt activity.
Effects of cisplatin on Akt activation and Bad phosphorylation.

Fig. 3. Kinase-deficient Akt sensitizes Caov-3 and A2780 cells to cisplatin. A, empty vector (CMV-6), wild-type Akt, or AktK179M-expressing Caov-3 cells grown in 100-mm dishes were treated with 1 mM cisplatin for 3 h. The lysate samples were immunoprecipitated (I.P.) with anti-HA antibody (A-HA). For analysis of the level of ectopically expressed Akt protein products (bottom row), immune complexes were precipitated with protein A-Sepharose, and the kinase reaction was carried out in the presence of cold ATP and GSK-3 fusion protein, as described in “Materials and Methods.” Cell viability was assessed in empty vector- (A), wild-type Akt- ( ), and AktK179M-expressing ( ) Caov-3 (B and D) and A2780 (C) cells after treatment with the indicated concentrations of cisplatin (B and C) and transplatin (D) as described in “Materials and Methods.”

Table 1 Effect of AktK179M sensitization of Caov-3 or A2780 cells to cisplatin-induced cytotoxicity

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>AktK179M-expressing</th>
<th>Cisplatin sensitization</th>
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<tr>
<td>Caov-3</td>
<td></td>
<td></td>
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<tr>
<td>Parental</td>
<td>380 ± 25</td>
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<td>A2780</td>
<td>84 ± 4</td>
<td>57 ± 2</td>
<td>1.5</td>
</tr>
<tr>
<td>Empty vector CMV-6</td>
<td>78 ± 3</td>
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* Parental and empty vector cells were analyzed in parallel and with equal concentrations of cisplatin and transplatin in the range 0–1 mM in quadruplicate. Transplatin had no effect on the viability of any cells.

* Sensitization is defined as the ratio of the IC50 value for the empty vector cells to the IC50 value for the AktM179M-expressing cells.

(Fig. 3A, top row). The viability of Caov-3 cells was not affected by increasing concentrations of cisplatin of >100 μM. Further titrations revealed IC50 values of 380 and 422 μM for parental and empty vector-expressing Caov-3 cells, respectively (Table 1). In contrast, the AktK179M-expressing Caov-3 cells exhibited an IC50 as low as 84 μM, indicating a >5.2-fold greater sensitivity to cisplatin than the empty vector-expressing Caov-3 cells (Fig. 3B and Table 1). On the other hand, the IC50 value of A2780 cells was 84 μM (Table 1). The AktK179M-expressing A2780 cells exhibited IC50 values of 57 μM, indicating a >1.5-fold greater sensitivity to cisplatin than the empty vector-expressing A2780 cells (Fig. 3C and Table 1).

Transplatin had no discernible effect on the AktK179M-expressing Caov-3 cells at concentrations at which the viability after treatment with cisplatin was <40% (Fig. 3D). In further titrations, no significant effect was observed with transplatin even at 250 μM, indicating that the requirement for sensitization by kinase-deficient Akt depends on the stereospecific DNA-binding properties of cisplatin, consistent with the results for the activation of Akt (Fig. 1C). Expression of wild-type Akt did not affect the sensitivity to cisplatin compared with the control line (Fig. 3, B and C). Thus, the sensitization to cisplatin observed in the kinase-deficient Akt-expressing cells appeared to be attributable to interference with activated Akt. We confirmed these results with other clonal derivatives of Akt (data not shown).

Interference of phosphorlyated Bad at Ser-112 or Ser-136 sensitizes Caov-3 and A2780 cells to cisplatin but not transplatin. We next examined whether the phosphorylation of BAD is also required for cell viability after cisplatin treatment of Caov-3 (Fig. 4B) and A2780 cells (Fig. 4C). To test the importance of BAD Ser-112 and Ser-136 for cell viability, we transfected Caov-3 and A2780 cells with mutant BAD constructs in which Ser-112 (BADS112A), Ser-136 (BADS136A), or both (BADS2A) were converted to alanine so that BAD could no longer be phosphorylated at these sites (12). We first confirmed the overexpression of ectopically expressed BAD protein products (Fig. 4A, bottom row) and the negative effects of the expression of BAD112A, BAD136A, or BAD2A on BAD phosphorylation (Fig. 4A, top row). BADS112A-, BADS136A-, and BAD2A-expressing Caov-3 cells exhibited IC50s of 77, 78, and 56 μM, respectively, indicating more than 6.0-, 5.9-, and 8.4-fold greater sensitivity, respectively, to cisplatin than the empty vector (pCDNA3)-expressing Caov-3 cells (Fig. 4B and Table 2). On the other hand, BADS112A-, BADS136A-, and BAD2A-expressing A2780 cells exhibited IC50s of 52, 53, and 36 μM, indicating more than 1.5-, 1.5-, and 2.3-fold greater sensitivity, respectively, to cisplatin than the empty vector (pCDNA3)-expressing A2780 cells (Fig. 4C and Table 2).

Expression of wild-type BAD did not affect the sensitivity to cisplatin compared with the empty vector (pCDNA3)-expressing control lines (Fig. 4, B and C). Interestingly, exogenous expression of either BAD112A or BAD136A in Caov-3 and A2780 cells decreased the viability after treatment with cisplatin to a level intermediate between that of cells expressing endogenous wild-type BAD and cells expressing BAD2A. Transplatin had no discernible effect on the BAD112A-, BAD136A-, or BAD2A-expressing Caov-3 cells at concentrations at which the viability after treatment with cisplatin was <40% (Fig. 4D). Thus, the sensitization to cisplatin observed in the mutant BAD-expressing cells appeared to depend on the ste-
Fig. 4. Both BADS112A and BAD136A sensitize Caov-3 and A2780 cells to cisplatin. A, empty vector (pCDNA3)-, wild-type BAD-, BADS112A-, BADS136A-, or BAD2SA-expressing Caov-3 cells grown in 100-mm dishes were treated with 1 mM cisplatin for 3 h. The lysate samples were immunoprecipitated (IP) with anti-HA antibody (A-HA). Immune complexes were precipitated with protein A-Sepharose, and the isolated proteins were analyzed by electrophoresis on 8% SDS-polyacrylamide gels, followed by Western blotting with anti-BAD antibody for analysis of the level of ectopically expressed BAD protein products (bottom row) or with anti-phospho-BAD (Ser-112; middle row) or anti-phospho-BAD (Ser-136; top row) antibody for analysis of the effects of ectopically expressed BAD on BAD phosphorylation. Cell viability was assessed in empty vector (pCDNA3)-(○), wild-type BAD- (△), BADS112A- (●), BADS136A- (■), and BAD2SA-expressing (□) Caov-3 (B and D) and A2780 (C) cells after treatment with the indicated concentrations of cisplatin (B and C) and transplatin (D) as described in “Materials and Methods.”

Respecific DNA-binding properties of cisplatin, which is consistent with the results for the phosphorylation of BAD (Fig. 2), and the sensitization appeared to be attributable to interference with BAD phosphorylated at Ser-112 and Ser-136. We confirmed this result with other clonal derivatives of BAD (data not shown).

Effect of Cisplatin on Phosphorylation of BAD in Kinase-deficient Akt-expressing Cells. To examine whether Akt acts upstream of BAD, we examined whether cisplatin induces the phosphorylation of BAD in kinase-deficient Akt (AktK179M)-expressing Caov-3 cells. Although cisplatin induced the phosphorylation of BAD at Ser-112 in empty vector (CMV6)-expressing cells, cisplatin did not induce the phosphorylation of BAD at Ser-136 in the kinase-deficient Akt (AktK179M)-expressing cells (Fig. 5B). Interestingly, the phosphorylation of BAD at Ser-112 induced by cisplatin was detected in both empty vector (CMV6)-expressing cells and kinase-deficient Akt (AktK179M)-expressing cells (Fig. 5A). On the other hand, cisplatin-induced Akt activity was not changed in cells transfected with BAD mutants (data not shown). These data confirm that Akt functions upstream of the phosphorylation of BAD at Ser-136.

DISCUSSION

This study showed that BAD is phosphorylated in cells with cisplatin-induced DNA damage and that BAD phosphorylation is required for cell viability after cisplatin treatment in both cisplatin-resistant and -sensitive cells. We used an Akt derivative rendered kinase-inactive by point mutation within the Akt catalytic domain (AktK179M) to block the activation of Akt. Caov-3 and A2780 cells expressing inactive Akt were sensitized to the cytotoxic effects of cisplatin compared with its effects on parental cells, on an empty vector-expressing control cell line, or on a line overexpressing wild-type Akt. In addition, we used BAD derivatives in which Ser-112 (BADS112A), Ser-136 (BADS136A), or both (BAD2SA) were converted to alanine to block the phosphorylation of BAD. Caov-3 and A2780 cells expressing BADS112A, BADS136A, or BAD2SA were sensitized to the cytotoxic effects of cisplatin compared with its effects on parental cells, on an empty vector-expressing control cell line, or on a line overexpressing wild-type BAD. The effect of overexpressed wild-type Akt or BAD on the viability of the cells treated with cisplatin was not significant, as was also seen in the case of cells in which survival signaling was modulated (12, 14, 32). Although the reasons for the lack of a significant effect on the viability of the cells overexpressing wild-type Akt or BAD is unknown, it is possible that endogenous expression of Akt or BAD might mask the effect of ectopically expressed wild-type Akt or BAD. Whereas the phosphorylation of BAD at Ser-136 was blocked by the treatment of cells with wortmannin, its phosphorylation at Ser-112 was blocked by treatment of the cells with an MEK inhibitor, PD98059. Moreover, although cisplatin-induced BAD phosphorylation at Ser-136 was blocked in a line overexpressing kinase-deficient Akt, its phosphorylation at Ser-112 was not blocked. These results suggest that cisplatin-induced DNA damage induces the phosphorylation of both BAD Ser-112 and Ser-136 via a MEK cascade and BAD Ser-136 via an ERK cascade and BAD Ser-112 via a MEK cascade.

Table 2 Effect of BAD mutant sensitization of Caov-3 or A2780 to cisplatin-induced cytotoxicity

<table>
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<th>Control*</th>
<th>IC₅₀ (µM)</th>
<th>Sensitization*</th>
<th>IC₅₀ (µM)</th>
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<th>IC₅₀ (µM)</th>
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<tr>
<td>Caov-3</td>
<td>418 ± 38</td>
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<td>36 ± 2</td>
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* Empty vector cells were analyzed in parallel and with equal concentrations of cisplatin and transplatin in the range 0–1 mM in quadruplicate. Transplatin had no effect on the viability of any cells.

* Sensitization is defined as the ratio of the IC₅₀ values for the empty vector cells to the IC₅₀ value for the BAD mutant-expressing cells.
a PI-3K-PKB/Akt cascade, and that inhibition of either of these cascades sensitizes ovarian cancer cells to cisplatin.

PI-3K signaling is very important in mitogenesis, protein synthesis, membrane ruffling, and cell-cycle progression (33, 34). Sequence homology studies have revealed that the PI-3K domain is present in a variety of proteins active in DNA repair (35). In addition, it has been shown that wortmannin induces marked radiosensitivity in murine fibroblasts and human tumor cells and blocks the induction of p53 after DNA damage (36). Thus, PI-3K signaling is suspected to be involved in DNA repair. The PI-3K-Akt-BAD cascade is reported to suppress the apoptotic death induced by a variety of stimuli, including growth factor withdrawal, cell-cycle discordance, and loss of cell adhesion in a number of cell types (5–11). However, until recently there had not been any studies addressing the role of the PI-3K-Akt-BAD cascade in the DNA repair in cells treated with chemotherapeutic DNA-damaging drugs. This is the first report showing that cisplatin induces the phosphorylation of BAD at Ser-112 and Ser-136 (14). The presence of two phosphorylation sites in BAD may be necessary for DNA repair in human ovarian cancer cells treated with cisplatin.

Resistance to cisplatin is a multifactorial phenomenon, the elements of which may be placed in three general categories: (a) reduced accumulation of cisplatin, (b) elevated levels of glutathione and metallothionein, and (c) increased DNA damage tolerance or repair (37–40). Because cisplatin acts by forming DNA-DNA cross-links (both intrastrand and interstrand) and DNA-protein cross-links, resulting in DNA damage, the repair of the affected DNA clearly is an important mechanism of resistance to cisplatin (41). However, the mechanism of this DNA repair is not completely clear. In general, drug-induced apoptosis is dependent on the balance between cell cycle checkpoints and DNA repair mechanisms (42). Thus, the intracellular signaling that modulates apoptosis may be involved in DNA repair and may be an appropriate target for strategies to overcome the resistance to chemotherapeutic DNA-damaging drugs. This study showed that cisplatin treatment led to activation of Akt and phosphorylation of BAD, and that overexpression of kinase-deficient Akt or BAD caused sensitization to the cytotoxic effects of cisplatin in both sensitive and resistant cells. Thus, the activation of these cascades by cisplatin and the related function of these cascades appeared to be not very different between sensitive and resistant cells, as is also the case in the activation of the ERK and JNK cascades by cisplatin (18). The reason that Caov-3 is cisplatin-resistant and A2780 is cisplatin-sensitive is unclear. However, both Akt-dependent phosphorylation of BAD Ser-136 and ERK-dependent phosphorylation of BAD Ser-112 seemed to be necessary for maintaining cell viability following the genotoxic stress of cisplatin, leading to resistance to cisplatin in the case of sensitive cells and greater resistance to cisplatin in the case of resistant cells; therefore, blockage of these cascades may be the candidate for strategies to reverse the resistance to cisplatin.

BAD function is modulated by phosphorylation at two sites, Ser-112 and Ser-136 (14). The presence of two phosphorylation sites in BAD also suggests that the simultaneous activation of different survival pathways may result in the concomitant phosphorylation of BAD Ser-112 and Ser-136 by different kinase cascades (12). Akt primarily triggers BAD phosphorylation at Ser-136, and phosphorylation at this site is sufficient to promote survival in cerebellar granule cells (12). In other cell types, such as interleukin-3-dependent hematopoietic cells, prevention of cell death may involve both BAD Ser-112 and BAD Ser-136 (14). Thus, the role of BAD Ser-112 is different, depending on the type of cell or extracellular stimulus. Moreover, conflicting evidence regarding the potential role of MEK-dependent BAD phosphorylation has emerged in recent years. MEK inhibition had no effect on platelet-derived growth factor-induced BAD phosphorylation (12). In contrast, MEK inhibition was found to inhibit both interleukin-3 and granulocyte macrophage colony-stimulating factor-dependent BAD phosphorylation but to have no effect on cell survival (19). It has been reported very recently that the MAPK-activated pp90-ribosomal S6 kinase family catalyzed the phosphorylation of BAD Ser-120 (20), and that the Ras-MAPK pathway was involved in the phosphorylation of BAD Ser-112 (21, 22) and its function related to dissociation of BAD from Bcl-XL (21). Cisplatin induced the phosphorylation of BAD at both Ser-112 and Ser-136 (Fig. 2). Whereas cisplatin-induced phosphorylation of BAD Ser-112 was MEK-dependent, cisplatin-induced phosphorylation of BAD Ser-136 was PI-3K-Akt-dependent (Figs. 2 and 5). In addition, cisplatin-induced phosphorylation of both BAD Ser-112 and Ser-136 was involved in maintaining cell viability after cisplatin treatment (Fig. 4).

Thus, our results suggest that the ERK and PI-3K-Akt signaling cascades converge at BAD to suppress the apoptotic effect of BAD.

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We thank Drs. Michael E. Greenberg and Sandeep Robert Datta for the gift of the vectors encoding the various HA-tagged forms of Akt, either wild-type or kinase-dead (K179M mutant), and the various forms of HA-tagged BAD, either wild-type (pCDNA3-BAD) or BAD mutants (pCDNA3-BAD112A, pCDNA3-BADS136A, pCDNA3-BAD112A, and pCDNA3-BAD S to A at 112 and 136).

REFERENCES

EFFECTS OF CISPLATIN ON AKT ACTIVATION AND BAD PHOSPHORYLATION


Inhibition of BAD Phosphorylation Either at Serine 112 via Extracellular Signal-regulated Protein Kinase Cascade or at Serine 136 via Akt Cascade Sensitizes Human Ovarian Cancer Cells to Cisplatin

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