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

Jun Hayakawa, Masahide Ohmichi, Hirohis Kurachi, Yuki Kanda, Koji Hisamoto, Yukihiko Nishio, Kazushige Adachi, Keiichi Tasaka, Toru Kanzaki, and Yuji Murata

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 MAP2 kinase cascade with survival in PC-12 cells (1). Another signaling pathway requiring PI-3K activity was associated with antiapoptotic regulatory cascades (12, 13). Whereas BAD can be phosphorylated at either BAD at Ser-112 and Ser-136 sites. Whereas the phosphorylation of BAD at Ser-112 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 at a degree intermediate between that caused by exogenous expression of wild-type BAD and doubly substituted BAD2SA. 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.

MATERIALS AND METHODS

Materials. Wortmannin was purchased from Sigma Chemical Co. (St. Louis, MO). Genetincin 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 anti-phospho-ERK (Ser-202) and (Thr-204) antibody, were obtained from New England Biolabs (Beverly, MA). Rabbit polyclonal anti-HA antibody was obtained from Santa Cruz Biotechnology.
Inc. (Santa Cruz, CA). The Cell Titer 96-cell proliferation assay kit was obtained from Promega (Madison, WI).

**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% O₂ and 5% CO₂.

** 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-BADS120A), and pCDNA3-BAD 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.

**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 3 h. The lysate samples were immunoprecipitated with anti-AKT antibody. Immune complexes were precipitated with protein A-Sepharose, and the kinase reaction was carried out in the presence of cold ATP and GSK-3α/β 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 lysate samples were immunoprecipitated with phospho-BAD (Ser-136) antibody, and the isolated proteins were analyzed by electrophoresis on 8% SDS-PAGE. Transfer to nitrocellulose, Western blotting with 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 lysate samples was 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 lysate samples were immunoprecipitated with anti-HA antibody. Immune complexes were precipitated with protean A-Sepharose, and the isolated proteins were resolved by 8% SDS-PAGE, followed by Western blotting with phospho-BAD (Ser-112) and phospho-BAD (Ser-136) antibody, and washing were performed as described elsewhere (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.
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...
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 Phosphorylated 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 (BAD112A), Ser-136 (BAD136A), or both (BAD2SA) 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 BAD2SA on cisplatin phosphorylation (Fig. 4A, top row). BAD112A-, BAD136A-, and BAD2SA-expressing Caov-3 cells exhibited IC50 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, BAD112A-, BAD136A-, and BAD2SA-expressing A2780 cells exhibited IC50 values 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) control (Figs. 4B and 4C). 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 exogenous wild-type BAD and cells expressing BAD2SA. Transplatin had no discernible effect on the BAD112A-, BAD136A-, or BAD2SA-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-

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IC50 (µM)</th>
<th>AktK179M-expressing</th>
<th>Cisplatin sensitization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caov-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parental</td>
<td>380 ± 25</td>
<td>84 ± 2</td>
<td>5.2</td>
</tr>
<tr>
<td>Empty vector CMV-6</td>
<td>422 ± 32</td>
<td></td>
<td></td>
</tr>
<tr>
<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>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 AktM179K-expressing cells.

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**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-Akt antibody. For analysis of the level of ectopically expressed Akt protein products (bottom row), 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-Akt antibody. For analysis of the effects of ectopically expressed Akt on Akt activity (top 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- (C), wild-type Akt- (A), and AktK179M-expressing (B) 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.”

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(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

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**Fig. 4. Interference of Phosphorylated BAD at Ser-112 or Ser-136 Sensitizes Caov-3 and A2780 Cells to Cisplatin but not Transplatin.** (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-Akt antibody. For analysis of the level of ectopically expressed Akt protein products (bottom row), 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-Akt antibody. For analysis of the effects of ectopically expressed Akt on Akt activity (top 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- (C), wild-type Akt- (A), and AktK179M-expressing (B) 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.”

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(A2-HA). For analysis of the level of ectopically expressed Akt protein products (bottom row), 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-Akt antibody. For analysis of the effects of ectopically expressed Akt on Akt activity (top 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- (C), wild-type Akt- (A), and AktK179M-expressing (B) 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.”
reospecific 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-136 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 an ERK cascade and BAD Ser-136 via a MAPK cascade.

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

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Control</th>
<th>IC50 (µM)</th>
<th>Sensitization</th>
<th>IC50 (µM)</th>
<th>Sensitization</th>
<th>IC50 (µM)</th>
<th>Sensitization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caov-3</td>
<td>Empty vector pCDNA3</td>
<td>418 ± 38</td>
<td>77 ± 2</td>
<td>6.0</td>
<td>78 ± 3</td>
<td>5.9</td>
<td>56 ± 2</td>
</tr>
<tr>
<td>A2780</td>
<td>Empty vector pCDNA3</td>
<td>79 ± 3</td>
<td>52 ± 2</td>
<td>1.5</td>
<td>53 ± 2</td>
<td>1.5</td>
<td>36 ± 2</td>
</tr>
</tbody>
</table>

* 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 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-activating p99-ribosomal S6 kinase family catalyzed the phosphorylation of BAD Ser-112 (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.

ACKNOWLEDGMENTS

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-BADs112A, pCDNA3-BADs112A, and pCDNA3-BAD S to A at 112 and 136).

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