p53 Is a Determinant of X-Linked Inhibitor of Apoptosis Protein/Akt-Mediated Chemoresistance in Human Ovarian Cancer Cells

Michael Fraser, Brendan M. Leung, Xiaojuan Yan, Han C. Dan, Jin Q. Cheng, and Benjamin K. Tsang

Abstract

We established previously that X-linked inhibitor of apoptosis protein (Xiap) is a determinant of cisplatin (CDDP) resistance in human ovarian cancer cells and that down-regulation of Xiap sensitizes cells to CDDP in the presence of wild-type p53. Furthermore, Xiap up-regulates the phosphatidylinositol 3'-kinase/Akt pathway by increasing Akt phosphorylation. However, the precise relationships among Xiap, Akt, and p53 in chemoresistance are unknown. Here we show that both Xiap and Akt can modulate CDDP sensitivity individually but that Xiap requires Akt for its full function. Furthermore, dominant-negative Akt sensitizes ovarian cancer cells to CDDP (10 μM), an effect that is absent in cells expressing mutant p53 or treated with the p53 inhibitor pifithrin-α-hydrobromide (30 μM) but restored by exogenous wild-type p53. CDDP increased p53, decreased Xiap content, and induced apoptosis in OV2008 cells but not in the resistant counterpart (C13*). However, dominant-negative Akt restored all of these characteristics to C13* cells. Expression of a constitutively active Akt2 prevented CDDP-mediated down-regulation of Xiap and apoptosis in A2780cs cells. Akt2-mediated chemoresistance could not be reversed by Xiap down-regulation. These results suggest that whereas Xiap, Akt2, and p53 are important mediators of chemoresistance in ovarian cancer cells, Akt2 may be an important regulator of both Xiap and p53 contents after CDDP challenge. Inhibition of Xiap and/or Akt expression/function may be an effective means of overcoming chemoresistance in ovarian cancer cells expressing either endogenous or reconstituted wild-type p53.

Introduction

Although chemotherapy remains a major treatment modality for human ovarian cancer, chemoresistance is a clinical problem that severely limits treatment success. CDDP is a first-line chemotherapeutic agent in the treatment of ovarian cancer. It is now widely accepted that the apoptotic capacity of the cancer cell is pivotal in determining the response to chemotherapeutic agents. Indeed, several gene products that regulate apoptosis, i.e., p53, Akt, and PI3K, are frequently altered in cancer cells (1, 2). These observations suggest that inhibition of apoptosis by survival proteins is a key step in the development of chemoresistance.

The inhibitors of apoptosis proteins, originally identified in baculovirus, are potent endogenous inhibitors of programmed cell death. Xiap is an inhibitor of the execution caspase-3 and -7 and directly suppresses the mitochondrial apoptotic pathway by inhibiting caspase-9 (3). Our laboratory has demonstrated previously that CDDP decreases Xiap protein level, induces Akt cleavage, and induces apoptosis in chemosensitive, p53 wild-type ovarian epithelial cancer cells (4). These responses were not observed in chemoresistant, p53 wild-type ovarian epithelial cancer cells (C13*). However, down-regulation of Xiap by adoviral antisense in C13* cells increased CDDP sensitivity (5). By contrast, Xiap down-regulation was unable to induce apoptosis or increase CDDP sensitivity in CDDP-resistant, mutant p53 ovarian cancer cells (A2780cp), suggesting that p53 status is a determinant of Xiap-mediated chemoresistance. Taken together, these results suggest that Xiap regulates chemosensitivity by protecting cells from CDDP-induced apoptosis and that Xiap may manifest some of its effects through modulation of a p53-mediated pathway. In addition, Xiap overexpression induces chemoresistance and Akt phosphorylation, indicative of Akt activation, effects that were attenuated by the PI3K inhibitor LY294002 (6). Together, these data suggest that Xiap-mediated chemoresistance may be caused in part by the activation of the PI3K/Akt pathway. It has been shown in a number of cell types that Akt1 (Akt), Akt2, and Akt3 promote cell survival and suppress apoptosis induced by a variety of stimuli. A major downstream target of these kinases is Bad, a proapoptotic member of the Bcl-2 family of apoptotic regulators (7–10). The enzymes also phosphorylate and inactivate members (FKHR/FKHR1) of the Forkhead transcription factor family (11, 12), which are involved in the regulation of FasL transcription. Studies have demonstrated alterations of Akt2 at the DNA or mRNA levels in 15–20% of human ovarian cancers as well as Akt2 activation and overexpression in primary ovarian carcinomas (1, 12). However, the relationship among Akt2, Xiap, and other mediators of chemoresistance is unknown.

In the current study, we demonstrate that both Xiap and Akt2 can modulate CDDP sensitivity but that Akt2 is involved in regulating Xiap content in the presence of CDDP. Furthermore, we demonstrate that functional p53 is absolutely required for the chemosensitizing effects of Xiap and/or Akt down-regulation. Overall, our results are consistent with the notion that modulation of Xiap or Akt function may be a useful therapeutic strategy in overcoming chemoresistance in tumors expressing wild-type p53 or in tumors supplemented with exogenous p53.

Materials and Methods

Reagents. RPMI 1640 or DMEM/F-12 (Life Technologies, Inc.) was used for cell cultures. Both media were supplemented with 10% fetal bovine serum, streptomycin (100 μg/ml), penicillin (100 units/ml), fungizone (0.625 μg/ml), and 1% nonessential amino acids (all from Life Technologies, Inc.). CDDP and DMSO were supplied by Sigma. PFT was obtained from Tocris Inc. Adenoviral constructs with Xiap-s, Xiap-as, wild-type p53, and LacZ cDNA were provided by Dr. Ruth Slack (Adenovirus Core Facility, Neuroscience Research Institute, University of Ottawa). Adenoviral construct containing HA-tagged,
triple-A mutated (K179A, T308A, and S473A), kinase-dead DN-Akt was a generous gift from Dr. Kenneth Walsh (Cardiovascular Research, St. Elizabeth’s Medical Centre, Boston, MA). All adenovirus stock solutions were CoCl2 purified; primary antibodies were anti-Xiap rabbit polyclonal IgG (Trevigen), anti-p53 mouse monoclonal IgG (Transduction Laboratories), anti-p21WAF1/CIP1 mouse monoclonal IgG (Cell Signaling), and anti-HA mouse monoclonal IgG (Roche). Appropriate dilutions were determined empirically using the manufacturer’s instructions as a starting point.

**Cell Culture.** CDDP-sensitive (OV2008 and A2780s) and -resistant (C13* and A2780cp) cell lines were cultured as reported previously (6). Before each experiment, 1.8 x 10^5 cells were plated on uncoated 6-well plates in the appropriate medium with 10% fetal bovine serum for 12–18 h for proper attachment.

**Creation of Stably Transfected Cell Lines.** A2780s cells were stably transfected with pcDNA3 vector (Invitrogen) containing constitutively active HA-tagged, myristoylated Akt2 or pcDNA3 alone as reported previously in Yuan et al. (14).

**Adenovirus Infection and CDDP Treatment.** After 12–18 h of plating, cells were infected with adenoviral Xiap-s, Xiap-as, wild-type p53, DN-Akt, and/or LacZ control at various viral doses (MOI) as reported previously (6). Total MOI was maintained constant for all treatment groups. Adenovirus infection efficiency (MOI = 5; 24 h) was >90%, as determined by a 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside-staining assay against LacZ construct-infected cells. Unless otherwise specified, 10 μM CDDP was dissolved in DMSO and added to the cells in culture medium with 2% fetal bovine serum 18 h for proper infection efficiency (MOI 5).

**Total MOI was maintained constant for all treatment groups. Adenovirus cells were infected with adenoviral Xiap-s, Xiap-as, wild-type p53, DN-Akt, Yuan HA-tagged, myristoylated Akt2 or pcDNA3 alone as reported previously in Sasaki.**

**Yuan et al. (14).**

**Results and Discussion**

**The Akt Pathway Is Involved in Xiap-Mediated Chemoresistance.** To form the basis on which our subsequent studies were carried out and to confirm our previous report that Xiap is implicated in CDDP resistance in ovarian cancer cells, CDDP-sensitive, p53 wild-type OV2008 cells were infected with adenovirus carrying Xiap-s cDNA or LacZ at various MOIs (MOI = 0, 1, and 5) for 48 h. Twenty-four h after infection, cells were treated with 10 μM CDDP or DMSO. Western blotting analysis demonstrated an increase in Xiap protein level with increasing Xiap-s dose. Infection with LacZ alone did not increase Xiap protein level (data not shown). Although DMSO induced similar cellular morphologies and apoptotic responses in both Xiap-s- and LacZ-infected groups, overexpression of Xiap (MOI = 5) before the addition of CDDP decreased apoptosis 2.5-fold compared with LacZ (P < 0.01). The protective effect of Xiap was also dose dependent, with Xiap-s at MOI = 5 showing a 1.6-fold decrease in apoptosis relative to the Xiap-s group at MOI = 1 (P < 0.05; Fig. 1A). To further assess the role of Xiap in chemoresistant cells, C13* cells were infected with adenovirus carrying Xiap-as cDNA at various MOIs (MOI = 0, 10, 20, 30, and 40) for a total of 48 h. At 24 h after infection, cells were treated with 10 μM CDDP or DMSO control. Western analysis confirmed down-regulation of Xiap by Xiap-as.
Although down-regulation of Xiap alone did not induce apoptosis, sensitivity toward CDDP increased significantly as a function of Xiap-as MOI ($P < 0.05$; Fig. 1B). These results confirm the involvement of Xiap in chemoresistance, as reported previously by our laboratory (4–6).

In addition, our laboratory has reported previously that overexpression of Xiap increases Akt phosphorylation and renders ovarian cancer cells resistant to the cytotoxic actions of CDDP, an effect that is partially attenuated by LY294002 (6). Thus, to determine the role, if any, of Akt in Xiap-mediated chemoresistance, chemosensitive ovarian cancer cells (A2780s and OV2008) were infected with DN-Akt (MOI = 0, 10, 20, and 40). The dominant-negative effects of this Akt mutant have been demonstrated previously (15). At 1 h after infection, Xiap-s (MOI = 5) was added to each group (total MOI was equalized with LacZ). The cells were treated with 10 $\mu M$ CDDP or DMSO 48 h after infection. An extra group of cells infected with LacZ (MOI = 45) was included as a control. CDDP (10 $\mu M$) induced a significant apoptotic response ($P < 0.01$) in both A2780s (Fig. 2A) and OV2008 cells (Fig. 2B) when infected with LacZ (MOI = 45). Xiap-s induced resistance toward CDDP compared with LacZ control in both A2780s ($P < 0.01$) and OV2008 ($P < 0.05$) cells. In the absence of CDDP, replacement of LacZ with DN-Akt in A2780s cells induced apoptosis in a dose-dependent manner (MOI = 0, 10, 20, and 40) despite the presence of Xiap-s (MOI = 5; data not shown). However, no significant change in apoptotic response was observed in OV2008 under the same conditions, suggesting that the A2780s cell line is more dependent on the integrity of Akt for its survival than the OV2008 cell line. However, in both A2780s and OV2008 cells, replacement of LacZ with DN-Akt attenuated the protective effect of Xiap. CDDP-induced apoptotic response was restored up to 70% of control (LacZ; MOI = 45) in cells coexpressing Xiap (MOI = 5) and DN-Akt (MOI = 40). Taken together, these results support our hypothesis that Xiap is a determinant of CDDP sensitivity in ovarian cancer cells and that Akt is a downstream intermediate of Xiap-mediated chemoresistance.

**Akt Is a Determinant of Chemoresistance.** A recent study has demonstrated that an ovarian cancer cell line expressing a constitutively active PI3K catalytic subunit p110$\alpha$ is resistant to paclitaxel, relative to the parental control cells, an effect that was reversed by the PI3K inhibitor LY294002 (16). Moreover, data from our own laboratory have demonstrated that Xiap-mediated chemoresistance is partially attenuated by LY294002 (6). In addition, a recent time course study has demonstrated that expression of an active Akt2 in ovarian cancer cells renders the cells resistant to CDDP (14). In investigating further the mechanisms of Akt-mediated chemoresistance, we have extended these observations with a concentration-response study using chemosensitive ovarian cancer cells (A2780s), stably transfected with pcDNA3 expression vector carrying constitutively active Akt2 (A2780s-AAkt2). Responses to CDDP (0, 2.5, 5, 10, and 20 $\mu M$) were compared with A2780s cells stably transfected with empty pcDNA3 vector (A2780s-PMH6). Although CDDP-induced apoptosis in a concentration-dependent manner in A2780s-PMH6 cells ($P < 0.01$), the presence of constitutively activated Akt2 reduced the sensitivity of cells toward CDDP ($P < 0.01$; Fig. 3A). These data are consistent with results demonstrated by Yuan et al. (14).

To further investigate the involvement of Akt in chemoresistance, p53 wild-type chemoresistant cells (C13*) were infected for 48 h with DN-Akt (MOI = 0, 10, 20, and 40). The DN-Akt construct was tagged with a HA epitope polypeptide, and the presence of HA signal on Western blot was indicative of DN-Akt expression. Forty-eight h after infection, cells were treated with 10 $\mu M$ CDDP or DMSO. A significant difference between the CDDP-treated group and control ($P < 0.01$) was observed. Furthermore, the effect of DN-Akt on the CDDP sensitivity of C13* cells was concentration dependent ($P < 0.05$; Fig. 3B). Taken together, these data confirm that Akt modulates the sensitivity of human ovarian cancer cells to CDDP.

**p53 Function Is Required For Sensitization to CDDP Through Suppression of Akt Activity.** Previous studies from our laboratory have established that Xiap down-regulation sensitizes chemoresistant, p53 wild-type (C13*) but not p53 mutant (A2780s/p53) ovarian cancer cells to CDDP (5). However, concomitant reintroduction of wild-type p53 induced apoptosis and permitted the CDDP-sensitizing effects of Xiap down-regulation in these p53 mutant cells. To determine whether p53 was also required for the chemosensitizing effects of Akt
activity down-regulation, A2780cp cells were infected with DN-Akt (MOI = 0, 10, 20, and 40) for 48 h, followed by subsequent treatment with 10 μM CDDP for 24 h. DN-Akt failed to sensitize A2780cp cells to CDDP (data not shown), suggesting that a wild-type p53 is required for the proapoptotic effects of down-regulation of Akt activity. To determine whether p53 status is indeed a determinant of Akt-mediated chemoresistance, A2780cp cells were coinfected with DN-Akt (MOI = 0–40) and wild-type p53 (MOI = 10). The presence of reconstituted wild-type p53 sensitized the cells to CDDP in a DN-Akt-dependent manner (P < 0.001, CDDP; P < 0.01, DN-Akt; P < 0.05, interaction; Fig. 4A). This effect was also dependent on the concentration of wild-type p53 when the DN-Akt dose was held constant (P < 0.0001, all effects; Fig. 4B). The combined results demonstrate that although suppression of Akt sensitizes chemoresistant cells to CDDP, this effect requires the presence of a wild-type p53.

To determine whether the involvement of Akt in Xiap-mediated chemoresistance depends on p53 function, we used the specific p53 inhibitor, PFT. The inhibitory effects of this compound on p53 function have been reported previously (17–19). OV2008 cells coinfected
with Xiap-s (MOI = 5) and DN-Akt (MOI = 0, 40, and 80) were incubated with PFT (0, 10, 20, and 40 μM) and CDDP (10 μM). In the absence of PFT, DN-Akt reversed Xiap-induced chemoresistance in a dose-dependent manner (P < 0.05). However, PFT dose-dependently attenuated the effects of DN-Akt on Xiap-induced chemoresistance up to 20 μM (P < 0.01; Fig. 5A). Interestingly, at 40 μM, PFT increased overall cell death in all groups, indicative of nonspecific PFT toxicity; we subsequently determined the maximum tolerable dose of PFT to be 30 μM (data not shown), and this concentration was used for all subsequent experiments. 

To further confirm the requirement of p53 in DN-Akt-mediated chemosensitization, A2780cp cells were infected with DN-Akt (MOI = 40) and wild-type p53 (MOI = 0, 5, 10, 20, and 40) and treated with 10 μM CDDP in the presence or absence of PFT (30 μM). Apoptosis was assessed by nuclear morphology. **+, P < 0.01 relative to DN-Akt infection without PFT. ###, P < 0.0001 relative to wild-type p53 (MOI = 0) + CDDP. 

To confirm that the actions of PFT were, at least in part, specific to p53, C13* cells were infected with DN-Akt (MOI = 0, 40, and 80) and wild-type p53 (MOI = 0, 5, 10, and 20) and treated with 10 μM CDDP in the presence and absence of PFT (30 μM). Although P21 content was undetectable in the absence of exogenous p53, reintroduction of wild-type p53 increased p21 content in a dose-dependent manner, an effect that was attenuated by the presence of PFT (Fig. 5C). Finally, there was a significant interaction between DN-Akt and P53 IN XIAP/akt-MEDiated CHEMORESISTANCE.
wild-type p53 ($P < 0.05$), suggesting that Akt down-regulation is more effective at sensitizing the cells to CDDP when p53 content is high.

Thus, wild-type p53 function is required for the proapoptotic effects of either Xiap down-regulation (5) or Akt suppression; these effects are inhibited by pharmacological inhibitors of p53 or by endogenous mutant p53 and stimulated by the introduction of wild-type p53. As such, modulation of Xiap and/or Akt function may be a viable option in overcoming chemoresistance in tumors expressing wild-type p53. As such, modulation of Xiap and/or Akt function may be a viable option in overcoming chemoresistance in tumors expressing wild-type p53.

Akt2 Protects Against CDDP-Induced Down-Regulation of Xiap. Previous studies from our laboratory have demonstrated that CDDP decreases Xiap content in chemosensitive, but not chemoresistant cells (20). Our finding that Akt regulates p53 content is consistent with the reports of Ogawara et al. (22), in which basal p53 levels could be controlled by Akt activation through activation, but not nuclear translocation, of MDM2. Similarly, we observed that the subcellular localization of MDM2 was Akt independent (data not shown). Whereas CDDP has previously been shown to increase p53 content (23), to our knowledge, our report represents the first to demonstrate that this process is negatively regulated by Akt. We are currently investigating the precise mechanisms by which Akt regulates the CDDP-mediated induction of p53.
sistant, human ovarian cancer cells (4). However, if and how Akt is implicated in this process are not known. In accordance with our earlier data (4), CDDP (0–10 μM) decreased Xiap content in a concentration-dependent manner in OV2008 cells but not in C13* cells (P < 0.05, all effects; Fig. 6A). This difference was most evident at 10 μM CDDP, in which OV2008 cells expressed only about 50% of basal (control) Xiap, compared with C13* cells, which showed no difference from the control cells (P < 0.001). However, when C13* cells were infected with DN-Akt (MOI = 80) and treated with 10 μM CDDP, Xiap was down-regulated in a DN-Akt-dependent manner (P < 0.01; Fig. 6B).

As mentioned, Akt2 has been demonstrated to modulate cellular CDDP sensitivity in ovarian cancer cells (14). To examine whether Akt2 could regulate Xiap content, we used the A2780s-AAkt2 (active Akt2) and A2780s-PMH6 (control) cell lines. Although CDDP (10 μM) induced significant apoptosis (P < 0.001, relative to control; Fig. 6E) and decreased Xiap content (60% decrease at 10 μM, P < 0.05 overall, relative to control; Fig. 6, C and E) in A2780s-PMH6 cells, it failed to elicit either effect in A2780s-AAkt2 cells (P < 0.0001 compared with A2780s-PMH6, all effects), suggesting a possible role for Akt2 in the protection of Xiap against CDDP-induced down-regulation.

To test whether this protection of Xiap plays a significant role in the antiapoptotic effects of Akt2 activation, we down-regulated Xiap using an Xiap-as (MOI = 40–48-h infection) in A2780s-AAkt2 cells, followed by treatment with 10 μM CDDP (24 h). Although Western blot analysis confirmed the down-regulation of Xiap by Xiap-as, CDDP-induced apoptosis was minimal and not significantly different in the two treatment groups (data not shown), suggesting that maintenance of Xiap content is a functionally minor downstream event in the antiapoptotic effects of Akt2 activation.

Thus, Akt2 regulates the CDDP-mediated down-regulation of Xiap. Interestingly, it has been reported that Xiap is an in vitro substrate for cleavage by a number of caspases, including caspase-3, -6, -7, and -8 (24). In the same study, it was reported that Fas-induced apoptosis was associated with cleavage of Xiap. Thus, it is possible that Xiap is cleaved by CDDP-dependent activation of caspases, an effect that is inhibited by Akt2. In such a case, the failure of CDDP to decrease Xiap content in chemoresistant cells may be secondary to aberrant regulation of the Akt2 pathway. Interestingly, Akt is known to phosphorylate and inactivate FKHRL1 and FKHR1 (11), both key regulators of FasL expression. Our laboratory has demonstrated previously that CDDP can up-regulate both Fas and FasL in OV2008 and A2780s but can only up-regulate Fas in C13* cells and cannot up-regulate Fas and FasL in A2780cp cells, suggesting that deregulation of this system may be an important determinant of chemoresistance in ovarian cancer cells (25).

Whether activation of the Fas/FasL system by CDDP is an important event in the subsequent down-regulation of Xiap and whether this phenomenon is Akt2-dependent remain unclear.

Xiap is also known to act as its own E3 ubiquitin ligase (26). It is possible, therefore, that aberrations in the ability of Xiap to become ubiquitinated and degraded in the 26S proteasome may underlie the observed maintenance of Xiap content in chemoresistant, but not chemosensitive, cells after CDDP challenge. Whether Akt2 modulates the process of Xiap ubiquitination is not known. However, it is clear that Akt2 is a central modulator of the cellular response to CDDP, both through its ability to inhibit the expression and function of proapoptotic proteins such as Bad and p53 and, interestingly, through its ability to protect antiapoptotic proteins such as Xiap from CDDP-induced down-regulation.

Note that Akt2 activation did not increase basal Xiap levels, as has been demonstrated in our laboratory in rat granulosa cells (27).

Moreover, down-regulation of Xiap did not attenuate Akt2-mediated chemoresistance. Thus, although Xiap is not required for the antiapoptotic effects of Akt2 activation, Akt2 is required for full implementation of Xiap-mediated chemoresistance. We are currently investigating the mechanism(s) by which Xiap is down-regulated in response to CDDP and by which Akt2 functions to inhibit this process.

In addition, our work provides evidence that reversal of chemoresistance can be achieved by inhibiting the function of either Xiap and/or Akt but that this process requires a functional p53 to be effective. It is likely, therefore, that suppression of Akt (or Xiap) sensitizes cells to p53-mediated apoptosis after CDDP challenge. Thus, in cells expressing mutant p53 or treated with PFT, Akt or Xiap, down-regulation has no effect on cell death because p53 is nonfunctional. In tumors expressing wild-type p53, suppression of Akt or Xiap could lead to reversal of chemoresistance. However, our data suggest that modulation of Akt activity may be a more effective mechanism of overcoming chemoresistance because Akt2 appears to control Xiap levels but can exert its antiapoptotic effects in the absence of Xiap.

Thus, our work has demonstrated that Akt is a key regulator of CDDP sensitivity, both through its ability to inhibit CDDP-induced apoptosis by itself and through its implication as a downstream intermediate of Xiap-mediated chemoresistance. Furthermore, we have shown that p53 is an important determinant of the response to Akt down-regulation in these cells, and we provide evidence that the increase in p53 content attributable to CDDP is negatively regulated by Akt. In addition, we show that Akt2 can regulate the effects of CDDP on Xiap content and that the failure of Xiap to be down-regulated by CDDP in chemoresistant cells is an Akt2-dependent phenomenon.

In conclusion, our work provides evidence that p53 is an important determinant of Akt-mediated chemoresistance and that Xiap and Akt are important, intimately related regulators of the cellular response to CDDP. Both p53 mutation and chemoresistance occur at extremely high frequencies in ovarian cancer. Our data suggest that overcoming chemoresistance by modulation of Xiap and/or Akt will, in some way, involve the restoration of p53 function in p53 mutant or null cells.

Acknowledgments

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