

# XIAP Regulates Akt Activity and Caspase-3-dependent Cleavage during Cisplatin-induced Apoptosis in Human Ovarian Epithelial Cancer Cells<sup>1</sup>

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## ABSTRACT

Chemoresistance is a major hurdle for successful cancer therapy. Although multiple mechanisms have been implicated to be involved in cisplatin resistance, recent evidence has suggested that X-linked inhibitor of apoptosis protein (XIAP) may be a key determinant in chemosensitivity in ovarian cancer. Cell fate is determined by a balance between cell survival and apoptotic signaling. Whereas phosphatidylinositol 3-kinase (PI 3-K) and XIAP are believed to be important cell survival factors in human ovarian surface epithelial cancer cells, if and how they interact to confer resistance to chemotherapy is not known. In the present study, we have investigated the role of XIAP in the regulation of the PI 3-K/Akt survival pathway in chemosensitive (A2780-s, OV2008, and OVCAR-3) and resistant (A2780-cp) ovarian cancer cell lines and the nature of this interaction in cell death/survival signaling. Cisplatin decreased XIAP protein levels and induced Akt cleavage and apoptosis in chemosensitive, but not in resistant, ovarian cancer cells. Cisplatin also induced cleavage of caspase-9 and caspase-3, a process blocked by XIAP overexpression. Pretreatment of ovarian cancer cells and their whole cell lysate with tetrapeptide inhibitors of caspases *in vitro* significantly decreased Akt cleavage induced by cisplatin and exogenous active caspase-3. Adenoviral sense XIAP cDNA expression increased XIAP protein levels and increased Akt phosphorylation, indicative of activation of Akt and, likely, of PI 3-K. This was associated with a decrease in cisplatin-induced apoptosis. In a cell line (OVCAR-3) where basal phosphorylated Akt levels were high, XIAP overexpression failed to increase further the level of this phosphoprotein. XIAP down-regulation induced Akt cleavage and apoptosis, and treatment of whole cell lysate with human recombinant active caspase-3 resulted in a similar pattern of Akt cleavage. In the presence of the PI 3-K inhibitor (LY294002), XIAP overexpression failed to block cisplatin-induced apoptosis and to induce Akt phosphorylation, suggesting that the site of action of XIAP is upstream of Akt in this cell survival pathway. Taken together, the results indicate that XIAP prevents apoptosis through a PI 3-K-dependent inhibition of the caspase cascade. These results demonstrate a novel mechanism by which XIAP regulates apoptosis and the possible involvement of the PI 3-K/Akt survival pathway in XIAP-mediated chemoresistance of ovarian cancer cells.

## INTRODUCTION

Akt is a serine/threonine protein kinase originally discovered as the cellular counterpart of the v-Akt transforming protein of a retrovirus (AKT8) that caused T-cell lymphomas in mice (1). It is also known as protein kinase B or Rac (2–4). Akt is an inactive cytosolic protein; it is recruited to the plasma membrane and activated by phosphorylation at threonine 308 and serine 473 in response to growth factors or

cytokines (5–9). The cell survival role of Akt has been demonstrated in multiple cell lineages (10–14).

The molecule responsible for the recruitment of Akt to the cellular membrane as well as activation of Akt is the lipid kinase PI 3-K.<sup>4</sup> PI 3-K phosphorylates phosphoinositides (PtdIns) at position 3 of the inositol ring. More recently, PI 3-K has emerged as an important signaling molecule for diverse growth factor receptors in a variety of cell types (15). Upon activation, PI 3-K induces Akt phosphorylation, which in turn phosphorylates and blocks the actions of Bad, a pro-apoptotic member of the Bcl-2 family (16), as well as altering the activity of a number of other pro-apoptotic mediators, including caspase-9, forkhead, and NFκB (10, 17, 18). The kinase Akt has been shown to induce phosphorylation of procaspase-9, suggesting that caspase-processing and apoptosis can be directly regulated by protein phosphorylation (17). This is supported by the observation that an activated form of Akt is also able to block apoptosis (13). The recent identification of a new oncogene derived from the regulatory subunit of PI 3-K (*p65-PI 3-K*) linked PI 3-K to mammalian tumor development (19). Increased PI 3-K has also been proposed to induce oncogenic transformation (19–21). More recently, another study revealed that *PIK3CA* gene (encoding the p110α subunit of PI 3-K) is a putative oncogene in ovarian cancer (22); this is supported by a similar observation in cervical cancer (23).

IAPs constitute a family of intracellular anti-apoptotic proteins that were first identified as analogues of genes present in baculovirus. It includes XIAP, HIAP-1, human HIAP-2, neuronal apoptosis inhibitory protein, and survivin (24, 25). These proteins are characterized by the presence of a caspase recruitment domain and an NH<sub>2</sub>-terminal baculovirus inhibitor of apoptosis repeat motif. With the exception of XIAP and survivin, they also include a COOH-terminal ring-zinc finger domain, believed to be involved in protein-protein and protein-nucleic acid interactions. Only a few reports to date have addressed the mechanisms of action of these anti-apoptotic proteins. XIAP has been shown to be a direct inhibitor of caspase-3 and caspase-7 (26) and to interfere with the Bax/cytochrome *c* pathway by inhibiting caspase-9 (27, 28). Overexpression of XIAP has been shown to protect Chinese Hamster Ovary and RAT-1 cells from menadione or growth factor withdrawal-mediated apoptosis (25), to suppress apoptosis in HeLa cells induced by transient transfection with interleukin 1β converting enzyme (29), and to suppress apoptosis induced by the Sindbis virus (30). These reports suggest that XIAP may play a key role in the regulation of apoptosis in cancer cells.

In the present study, we have used ovarian cancer cell lines as an *in vitro* model to study the role of the PI 3-K/Akt pathway in the anti-apoptotic action of XIAP and to examine its possible involvement in chemoresistance in human ovarian cancer.

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<sup>4</sup> The abbreviations used are: PI 3-K, phosphatidylinositol 3-kinase; PtdIns, phosphoinositides; PtdIns(3,4,5) P3, phosphatidylinositol (3,4,5) trisphosphate; IAP, inhibitor of apoptosis proteins; XIAP, X-linked IAP; HIAP, human IAP; DMEM:F12, DMEM:Ham's F-12; FBS, fetal bovine serum; Z-VAD-fmk, benzyl-oxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; DEVD-fmk, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone; MOI, multiplicities of infection; RT-PCR, reverse transcription-PCR; ILK, integrin-linked kinase.

## MATERIALS AND METHODS

**Reagents.** Cisplatin, Hoechst 33248, and LY294002 were obtained from Sigma (St. Louis, MO). Cisplatin solutions were prepared in the dark and used fresh each time. RPMI 1640, DMEM:F-12, and FBS were purchased from Life Technologies, Inc. (Burlington, Ontario, Canada). Human recombinant active caspase-3 and -9 were purchased from PharMingen Canada (Mississauga, Ontario, Canada). Polyclonal antihuman p85 PI 3-K antibody was a generous gift from Dr. Johné Liu (Loeb Health Research Institute, Ottawa, Canada). Antihuman PhosphoPlus Akt (Ser473) and Akt antibodies were obtained from the New England Biolabs (Mississauga). Z-VAD-fmk was purchased from BIOMOL (Plymouth Meeting, PA) and DEVD-fmk from Medical & Biological Laboratories (Nagoya, Japan). Adenoviral LacZ, antisense and sense XIAP constructs, and rabbit polyclonal antihuman XIAP antibody were generous gifts from Dr. Robert Korneluk (Apoptogen, Inc., Ottawa, Ontario, Canada). Rabbit polyclonal anti-XIAP antibody was raised against a glutathione *S*-transferase fusion protein that was expressed in *Escherichia coli* using the pGEX vector system (Pharmacia LKB) containing full-length XIAP cDNA and affinity-purified through glutathione-Sepharose. The antibody was affinity-purified by passing through a glutathione *S*-transferase-XIAP glutathione-Sepharose column. Specificity was confirmed using the depleted antibody passed through the affinity column on Western blots, and cross-reactivity with other IAPs was not noted.

**Cell Culture.** Human ovarian surface epithelial cancer cells were gifts from Dr. Rakesh Goel from the Ottawa Regional Cancer Center, Ottawa, Canada. Cells were cultured in 150-mm bottles at 37°C in an atmosphere of 5% CO<sub>2</sub> in either RPMI 1640 (OV2008 and OVCAR-3) or DMEM-F12 (A2780s and A2780-cp) containing 10% FBS, 1% nonessential amino acids, 0.5%

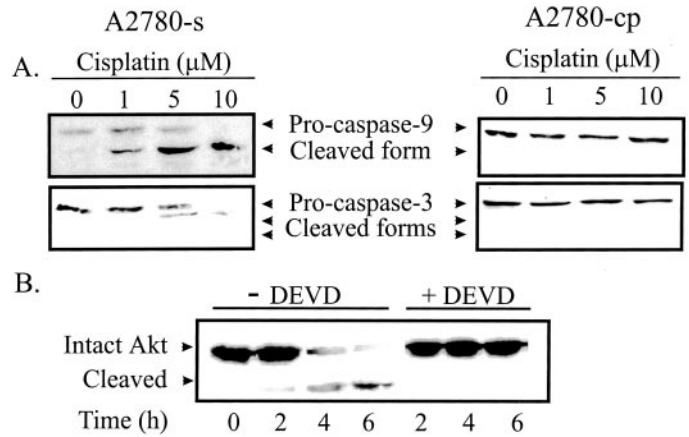


Fig. 2. Akt cleavage during cisplatin treatment is caspase-3 dependent. A. A2780-s and A2780-cp cells were treated as in Fig. 1B. A2780-s cell lysate was treated *in vitro* with human recombinant caspase-3 (10 μg) for 2, 4, and 6 h in the absence or presence of DEVD (25 μM). Akt cleavage was assessed by Western analysis.

streptomycin-penicillin, and 0.25% fungizone. Cells ( $1 \times 10^6$ ) were plated in log growth phase onto 60-mm dishes for 24 h in the above culture medium before the initiation of treatment.

**Experimental Design.** For cisplatin concentration-response studies, culture medium containing 10% FBS was replaced with FBS-free medium, and cells were cultured for 24 h in the presence of different concentrations of cisplatin (0, 1, 5, and 10 μM in DMF). For XIAP sense or antisense adenoviral infections using different MOI, culture medium containing 10% FBS was replaced with 0.5 ml of 10% FBS-medium containing adenoviral XIAP sense or antisense cDNA, and the cells were incubated initially for 1 h (with frequent shaking to ensure maximal infection) and then cultured in 2 ml of FBS-free medium for 24 h (antisense XIAP) or 48 h (sense XIAP). After 48 h of sense-XIAP infection, the medium was discarded, and cells were treated for 24 h in the absence or presence of cisplatin (10 μM) and/or 20 μM LY294002 (a specific PI 3-K inhibitor; Ref. 31). At the end of the culture period, floating [recovered by centrifugation ( $4000 \times g$  for 5 min)] and attached [subsequent to trypsin treatment (0.05% trypsin and 0.53 mM EDTA at 37°C for 3–5 min)] cells were pooled and centrifuged ( $4000 \times g$  for 5 min). For Hoechst staining, cell pellets were resuspended and fixed in 10% formalin (Fisher Scientific, Nepean, Ontario, Canada) containing Hoechst 33248 nuclear stain (12.5 ng/ml) and stored at 4°C for 24 h before being spotted onto slides for fluorescence microscopy. Total protein was extracted from cell pellets with a lysis buffer (pH 7.4) containing 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40 in PBS, protease inhibitor phenylmethylsulfonyl fluoride (1 mM), and 1 mM sodium orthovanadate. Total RNA was extracted for mRNA measurements using Trizol (Life Technologies, Inc.) according to the manufacturer's instructions.

**Hoechst Staining.** Nuclear staining was viewed and photographed using a Zeiss fluorescence microscope. Cells with typical apoptotic nuclear morphology (nuclear shrinkage, condensation, and fragmentation) were identified and counted using randomly selected fields on numbered photographic slides, with the "counter" unaware of the treatment, so as to avoid experimental bias. A minimum of 200 cells/treatment group were counted in each experiment.

**Protein Extraction and Western Analysis.** Cells were sonicated (10 s) in the lysis buffer, and sonicates were centrifuged ( $12,000 \times g$  for 20 min at 4°C) to remove insoluble material. The supernatant was recovered and stored at -20°C pending analysis. Protein content was determined with the Bio-Rad DC Protein Assay. Protein extracts (50 μg) were heated (94°C for 3 min), resolved by 10% SDS-PAGE and electrotransferred to nitrocellulose membranes (15 V for 30 min) using a semidry transfer (Bio-Rad, Mississauga, Ontario, Canada). The membranes were then blocked (2 h, room temperature) with PBS containing 5% milk powder, then incubated with anti-PI 3-K (1:8000), XIAP (1:2000), Akt (1:1000), or Phospho-PKB/Akt (1:1000) antibody (2 h, room temperature), and subsequently with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:5000; room temperature; for 45 min). Peroxidase activity was visualized with the enhanced chemiluminescence kit (Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

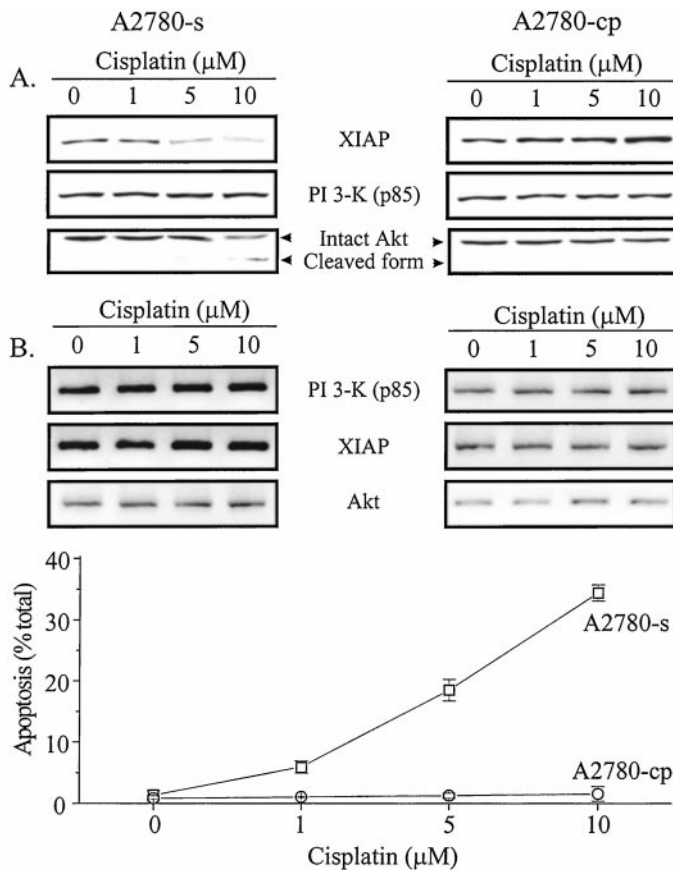
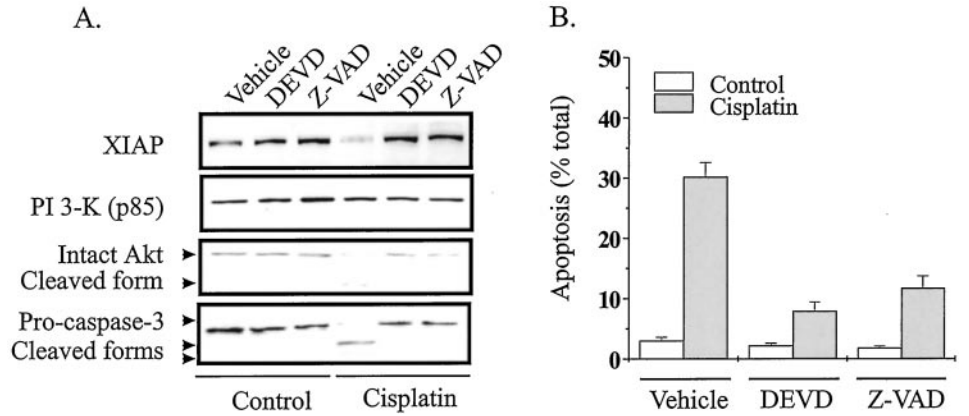


Fig. 1. Effect of cisplatin concentrations on XIAP, PI 3-K, and Akt mRNA and protein content of human ovarian cancer cell lines. Cells ( $1 \times 10^6$ ) were plated for 24 h in DMEM-F12-10% FBS and cultured for 24 h in serum-free DMEM-F12 with different cisplatin concentrations (0, 1, 5, and 10 μM). The same amount of vehicle (DMF) was added in each group. Cells were collected for Hoechst staining to count apoptotic cells. Total proteins and RNA were extracted for analysis by Western blot (A) and RT-PCR (B), respectively. Representative blots are presented. Data represent the mean  $\pm$  SE of four independent experiments.

Fig. 3. Effect of caspase inhibitors on cisplatin action in cultured human ovarian cancer cells. Two h before cisplatin treatment (10  $\mu$ M), cells were pre-treated with DEVD-fmk (25  $\mu$ M) or Z-VAD-fmk (50  $\mu$ M). After 24 h, cells were lysed, and proteins were analyzed by Western blotting.



**Semiquantitative RT-PCR Analysis.** To measure the abundance of PI 3-K (p85), Akt, and XIAP mRNA, primers were chosen as described below and tested with different primer concentrations and different cycles to avoid mRNA amplification near plateau and saturation. Conditions for final analysis were chosen when amplification of mRNA was in the middle of the exponential amplification phase for all cisplatin concentrations tested. Total RNA (400 ng) from each treatment group was used for the preparation of first-strand cDNA by reverse transcription. The RNA samples were incubated (65°C for 10 min) with 0.5  $\mu$ g oligo(deoxythymidine) primers in a final volume of 10  $\mu$ l. Samples were then incubated (37°C for 60 min) in 20  $\mu$ l of a reaction buffer (1 $\times$ ) containing DTT (10 mM), deoxynucleotide triphosphate (1.25 mM), and Muloney murine leukemia virus reverse transcriptase (200 units). The reaction volumes were brought up to 60  $\mu$ l with autoclaved water. A negative control was also included, using the same reaction mixture but without Muloney murine leukemia virus reverse transcriptase to ensure the absence of any contaminating genomic DNA in the RNA template.

Expression of the p85 regulatory subunit of the PI 3-K gene was determined by amplification of a 595-bp region of the human p85 PI 3-K cDNA sequence (1819–2413 bp; Ref. 32). Amplification was carried out using the antisense downstream sequence 5'-AAGTTGAACGAGTGGTTG GG-3' and the sense upstream sequence 5'-CGTGTGGTCATCATACAGCC-3'. Human Akt1 mRNA (58–1681 bp; Ref. 1) was amplified using sense primer 5'-GCCTGGGTC AAGAAGTCAA-3' and antisense primer 5'-CATCCCTCCAAGC-TATCGTC-3'. For XIAP, expression of the gene was determined by amplification of a 389-bp region of the XIAP cDNA sequence (973–1361 bp; Ref. 25), and the sequences of the primers were 5'-GAAGACCCCTGGGAA-CAACA-3' (sense) and 5'-CGCC TTAGCTGCTCTTCAGT-3' (antisense). Each reaction mixture (final volume: 50  $\mu$ l) contained 1 $\times$  buffer, reverse transcriptase template, or negative control (5  $\mu$ l), MgCl<sub>2</sub> (1.5 mM), deoxynucleotide triphosphate (0.2 mM), primers (10  $\mu$ M each), and Taq polymerase (1.5 units). The PCR cycling conditions (30 cycles) chosen were as follows: (a) 30 s at 94°C; (b) 1 min at 55°C; and (c) 1 min at 72°C; with a subsequent 10-min extension at 72°C. Reaction products were analyzed on 1.0% agarose gels. Bands were visualized by ethidium bromide staining.

**In Vitro Caspase Inhibition.** To assess the role of caspases in cisplatin-induced Akt cleavage, cells were preincubated for 2 h with Z-VAD-fmk (50  $\mu$ M) or DEVD-fmk (25  $\mu$ M) in serum-free medium and subsequently cultured for 24 h in the absence or presence of cisplatin (10  $\mu$ M).

**Caspases Cleavage Assay.** A2780-s cell lysate (50  $\mu$ g of total protein) was incubated for 0, 2, 4, or 6 h at 37°C in PIPES assay buffer {PIPES (20 mM), NaCl (100 mM), DTT (10 mM), EDTA (1 mM), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonic acid (0.1%; w/v), and sucrose (10%; w/v); pH 7.2} containing 10  $\mu$ g of recombinant active caspase-3 or -9 in the absence or presence of caspase-inhibitor DEVD (25  $\mu$ M). Total volume of the reaction was 40  $\mu$ l. The incubation was terminated with the addition of an equal volume of 2 $\times$  sample buffer [Tris-HCl (100 mM; pH 6.8), DTT (200 mM), SDS (4%; w/v), glycerol (20%; v/v), and bromophenol blue (0.2%)]. Akt cleavage was assessed by Western blotting.

**Statistical Analysis.** All experiments were repeated at least three times. Data were subjected to one- or two-way ANOVA (PRISM software version 2.0; GraphPad, San Diego, CA). Differences between experimental groups were determined by the Tukey test.

**RESULTS**

**Cisplatin Decreases XIAP Protein Content and Induces Akt Cleavage in Cisplatin-sensitive Ovarian Cancer Cells.** To test whether XIAP and PI 3-K/Akt pathway is involved in the action of cisplatin, ovarian cancer cells were cultured with different concentrations of cisplatin for 24 h (Fig. 1). Cisplatin significantly induced apoptosis in a concentration-dependent manner ( $P < 0.05$ ) in sensitive cells (A2780-s) and had no effect on the resistant variant (A2780-cp). Cisplatin reduced XIAP protein content and induced Akt cleavage, but had no effect on the levels of p85 regulatory subunit of PI 3-K (Fig. 1A). To determine whether the action of cisplatin was mediated through changes in gene expression, RT-PCR analysis were carried out (Fig. 1B). Cisplatin had no effect on the levels of mRNA transcripts of XIAP, PI 3-K (p85), or Akt.

**Akt Cleavage Is Mediated through Caspase-3 Activation.** Cleavage of caspase-3 and -9 was also observed in response to increasing concentrations of cisplatin in the cisplatin-sensitive cells (A2780-s; Fig. 2). To demonstrate the relationship between the cleavage of Akt and caspase-3 activation, A2780-s cell lysate was incubated for up to 6 h with human recombinant active caspase-3 in the

Fig. 4. Overexpression of XIAP in human ovarian cancer cells. Cells (1  $\times$  10<sup>6</sup>) were plated for 24 h in RPMI 1640 (OV2008 and OVCAR-3) or DMEM-F12 (A2780-s and A2780-cp) containing 10% FBS and infected (total viral concentration for all doses LacZ + XIAPs = 10 MOI) for 48 h with adenoviral sense XIAP (MOI = 0, 2.5, 5, and 10) and LacZ (control, MOI = 10, 7.5, 5, and 0) vectors, as described in "Materials and Methods." Cells were collected for Hoechst staining (for apoptotic cell count), and total proteins were extracted for Western analysis. Data represent the mean  $\pm$  SE of four independent experiments.

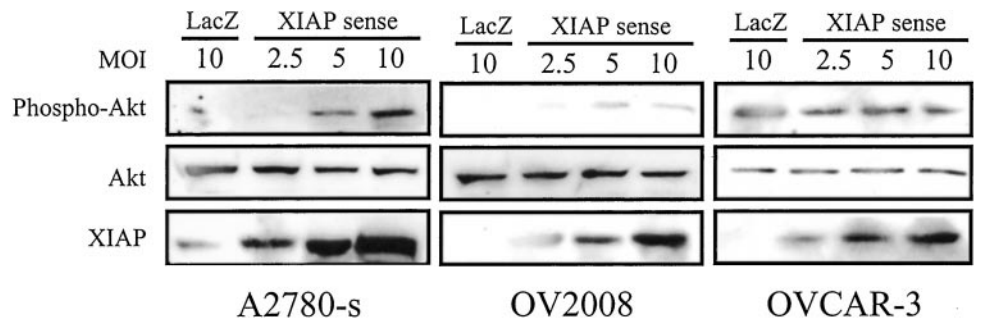
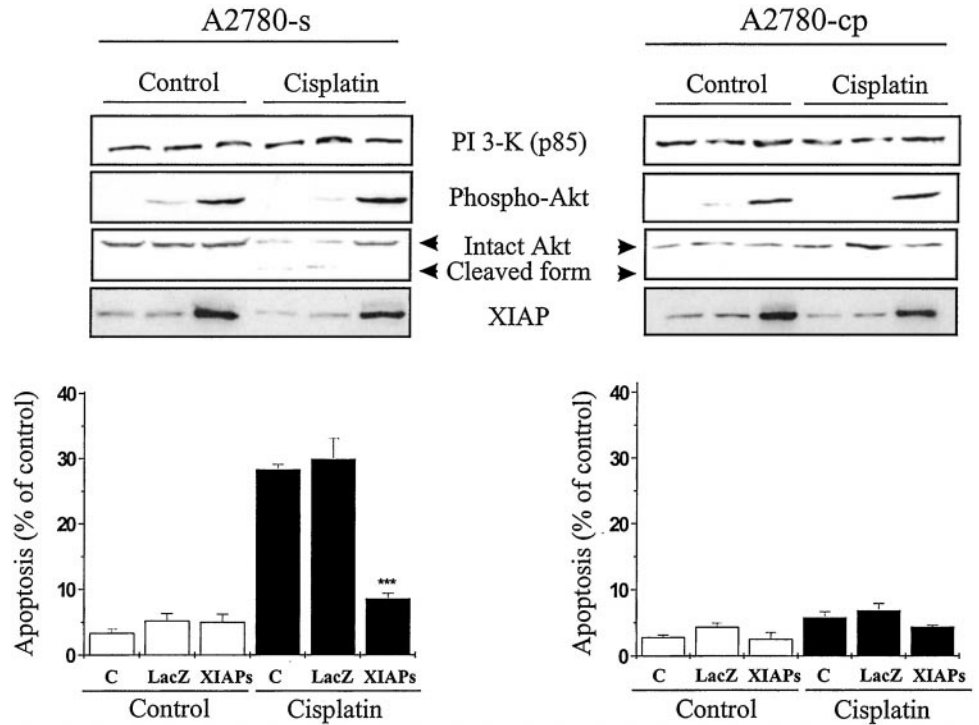


Fig. 5. Overexpression of XIAP in A2780-s and -cp cancer cells. Cells ( $1 \times 10^6$ ) were plated for 24 h in DMEM-F12-10% FBS and cultured for 48 h without virus (C) or infected for the same period with adenoviral sense XIAP (MOI = 10) or LacZ (control, MOI = 10) vectors, as described in "Materials and Methods." Cells were then treated for 24 h in the presence or absence of cisplatin ( $10 \mu\text{M}$ ) and collected for Hoechst staining (for apoptotic cell count). Total proteins were extracted for Western analysis. Data represent the mean  $\pm$  SE of four independent experiments.



absence or presence of the specific caspase-3 inhibitor (DEVD,  $25 \mu\text{M}$ ; Fig. 2B). The results demonstrate that active caspase-3 was able to cleave Akt *in vitro*, and this effect could be blocked by the caspase inhibitor. To demonstrate further the importance of caspase activation during cisplatin-induced apoptosis, A2780-s cells were cultured *in vitro* and treated with cisplatin ( $10 \mu\text{M}$ ) in the presence or absence of DEVD-fmk or Z-VAD-fmk, two-cell permeable caspase-3 inhibitors (Fig. 3). Addition of these inhibitors to the cell cultures attenuated the

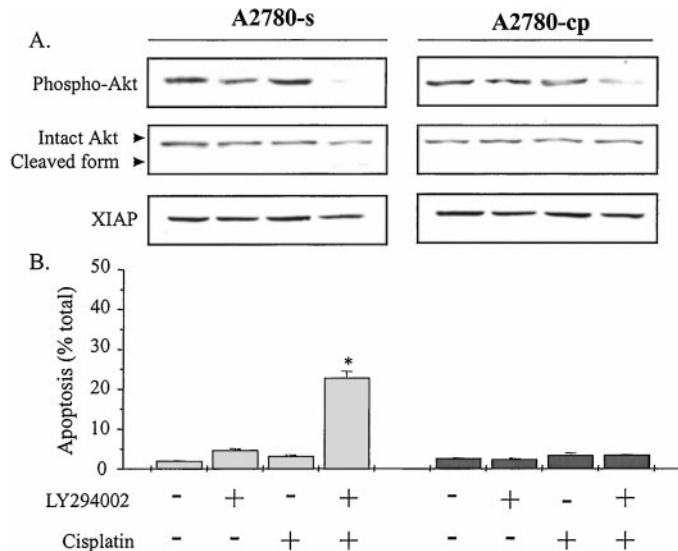


Fig. 6. LY294002 decreased phospho-Akt content and attenuated the protective effect of XIAP against cisplatin-induced apoptosis in ovarian cancer cells. A2780-s and A2780-cp ( $1 \times 10^6$ ), plated for 24 h in DMEM-F12-10% FBS, were infected with adenoviral sense XIAP (MOI = 10) for 48 h (as described in "Materials and Methods") and subsequently treated with LY294002 ( $20 \mu\text{M}$ )  $\pm$  cisplatin ( $10 \mu\text{M}$ ) for 24 h. At the end of the treatment period, cells were collected for Hoechst staining (for apoptotic cell count; B) and total proteins were extracted for Western analysis (A). Data represent the mean  $\pm$  SE of three independent experiments.

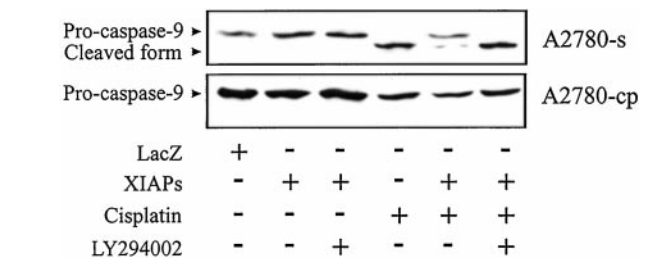


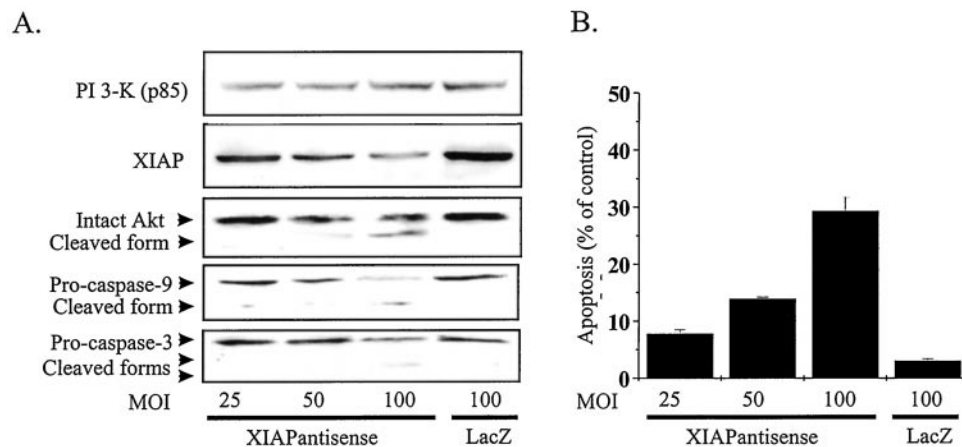
Fig. 7. Overexpression of XIAP blocks cisplatin-induced procaspase-9 cleavage. A2780-s and A2780-cp cells were infected for 48 h with adenoviral sense XIAP (MOI = 10) or LacZ vector (control), as described in "Materials and Methods," and treated with LY294002 ( $20 \mu\text{M}$ )  $\pm$  cisplatin ( $10 \mu\text{M}$ ) for 24 h. At the end of the treatment period, cells were collected and total proteins were extracted for Western blotting. Data represent the mean  $\pm$  SE of three independent experiments.

decrease in XIAP protein content and the cleavage of procaspase-3 and Akt as well as apoptosis ( $P < 0.05$ ) induced by cisplatin (Fig. 3).

**XIAP Overexpression Induces Akt Phosphorylation.** To characterize the relationship between XIAP and the PI 3-K/Akt pathway, we overexpressed XIAP protein in three different ovarian cancer cell lines using an adenoviral sense XIAP cDNA (Fig. 4). Overexpression of XIAP increased phospho-Akt contents in A2780-s and OV2008. However, basal phospho-Akt levels were high in OVCAR-3 cells and were not increased further with XIAP expression.

**XIAP Overexpression Blocks Cisplatin-induced Apoptosis.** XIAP overexpression induced Akt phosphorylation in cisplatin-sensitive (A2780-s) and resistant (A2780-cp) ovarian cancer cells (Fig. 5). Overexpression of XIAP blocked cisplatin-induced apoptosis in A2780-s cells, whereas PI 3-K p85 protein levels remained constant. To determine whether the XIAP-induced resistance of the cells to cisplatin might, in part, be attributable to an increased PI 3-K/Akt activity, we tested the effect of cisplatin in the presence of a PI 3-K inhibitor, LY294002 (Fig. 6A). In the presence of XIAP, LY294002 was sufficient to induce a small increase of apoptosis. The combination of LY294002 plus cisplatin induced a synergistic increase in

Fig. 8. The influence of XIAP down-regulation on p85 content, cleavage of pro-caspase-3, pro-caspase-9 and Akt, and apoptosis in A2780-s cancer cells. Cells ( $1 \times 10^6$ ) were plated for 24 h in DMEM-F12-10% FBS and infected (total viral concentration: MOI = 100) for 48 h with different doses of adenoviral antisense XIAP (MOI = 0, 25, 50, and 100) and LacZ (control, MOI = 100, 75, 50, and 0) vector, as described in "Materials and Methods." Cells were collected for Hoechst staining (for apoptotic cell count; B) and total proteins were extracted for Western analysis (A). Data represent the mean  $\pm$  SE of three independent experiments.



apoptosis in XIAP-overexpressing A2780-s cells. This increase in cell death was associated with a marked decrease in phospho-Akt levels. In the presence of LY294002 and cisplatin, phospho-Akt levels were also reduced in A2780-cp cells, but this was not sufficient to induce cell death. As shown in Fig. 7, XIAP overexpression also blocked cisplatin-induced procaspase-9 cleavage, an effect which was reversed in the presence of LY294002.

**XIAP Down-Regulation Induces Akt Cleavage and Apoptosis.** Expression of an adenoviral XIAP antisense cDNA in ovarian cancer cells (A2780-s) decreased XIAP content and induced apoptosis in an adenoviral concentration-dependent manner (Fig. 8). XIAP down-regulation also induced procaspase-9 and -3 cleavage as well as Akt cleavage.

## DISCUSSION

Ovarian epithelial cancer is the fifth leading cause of death among female cancers in America (33). Although chemotherapeutic agents, such as cisplatin, are widely used for the treatment of ovarian cancer, chemoresistance remains a major therapeutic problem, and the molecular mechanisms leading to chemoresistance are poorly understood. In the present study, we have demonstrated that cisplatin can induce apoptosis in a dose-dependent manner in sensitive (A2780-s), but not in resistant (A2780-cp), cell lines. The increased apoptosis was accompanied by a decrease in XIAP protein content but not in mRNA levels, suggesting that cisplatin regulates this anti-apoptotic factor at the protein level rather than by regulation of its gene expression. Although precisely how cisplatin decreases XIAP content in human ovarian cancer cells is not known, it has been shown to increase Fas and Fas ligand expression (34) and to activate downstream caspases.<sup>5</sup> Because XIAP is a substrate of caspase-3 and -7 (27), it is possible that the observed decrease in XIAP content could be a consequence of caspase-mediated processing after Fas activation. Alternatively, it is possible that the decrease in XIAP content subsequent to cisplatin challenge may be a consequence of proteasome-mediated processing, involving initially ubiquitination and subsequently degradation of the IAP molecule. In this context, it has recently been demonstrated that XIAP and HIAP-2 in thymocytes exposed to glucocorticoid or etoposide are selectively lost in a proteasome-dependent manner before death and that this process involves the autoubiquitination and degradation of the IAPs (35).

Although the physiological role of XIAP is not fully understood, it is considered an important intracellular anti-apoptotic factor and is known to suppress cell death by binding to and inhibiting caspase-3,

-7 (26), and -9 (27). In this regard, a decrease of XIAP levels could induce the apoptotic process, at least in part through activation of procaspases. These aspartate-specific proteases cleave and inactivate a number of cellular substrates during the induction of apoptosis (36). They constitute a point of no return in the activation of apoptotic processes (28), and the relative abundance of XIAP may be a key element in cell-fate determination. The mechanism(s) involved in the pro-apoptotic action of caspase-3 in ovarian cancer cells is not clear. It has been suggested that proteins involved in DNA breakdown (37), signal transduction (38, 39), and cytoskeletal proteins (40) may be putative physiological substrates for caspase-3. The action of caspase-3 involves the disruption of survival signals and/or proteins participating in the maintenance of cellular integrity. The present studies demonstrated that the survival factor Akt is cleaved during cisplatin-induced apoptosis and confirmed that this process is caspase-3-mediated. Overexpression of XIAP not only prevented cisplatin-induced Akt cleavage but increased phospho-Akt content. Akt is a protein kinase that promotes cell survival by inhibiting apoptosis, although precisely how it exerts its anti-apoptotic effects is not clear. It has recently been reported to directly phosphorylate and inactivate procaspase-9 as well as to block caspase-9-mediated apoptosis induced by cytochrome *c* via Apaf-1 in response to an early apoptotic stimuli (Ref. 17; reviewed in Ref. 41). Akt can phosphorylate BAD, resulting in the release of BCL<sub>2</sub> and inhibition of the apoptotic process. In addition, Akt can also promote cell survival by phosphorylating and inhibiting a forkhead transcription factor known to regulate Fas ligand gene expression (10).

To investigate the possible role of XIAP in the regulation of PI 3-K in ovarian cancer cells, we examined the influence of adenoviral sense XIAP cDNA expression on phospho-Akt content in the cisplatin sensitive cells A2780-s. XIAP overexpression increased phospho-Akt levels and suppressed cisplatin-induced apoptosis with no effect on the levels of the p85 subunit of PI 3-K, suggesting that XIAP may inhibit apoptosis in part through the activation, but not the expression/degradation, of PI 3-K. This notion was supported by the observation that inhibition of cisplatin-induced apoptosis with XIAP overexpression was reversed in the presence of LY294002, a PI 3-K inhibitor. The inhibition of PI 3-K activity, as evident by a decrease in phospho-Akt content, was sufficient to override the protective effect of XIAP on cisplatin-induced apoptosis. Although LY294002 also decreased phospho-Akt content in the XIAP-overexpressed, cisplatin-treated resistant variant A2780-cp, the levels of phospho-Akt were considerably higher than that in the sensitive cells under identical conditions, and apoptosis was not observed. The present studies have also shown that LY294002 alone induced only a slight decrease in phospho-Akt content as well as a small but nonsignificant increase in apoptosis.

<sup>5</sup> D. Schneiderman, C. W. Xiao, and B. K. Tsang, unpublished data.

Because the cells were treated with LY294002 for 24 h, and the latter has a relatively short half-life, it is possible that the decreased AKT phosphorylation could have been transient (*i.e.*, several hours after treatment). Taken together, these results suggest that the maintenance of a minimal level of phospho-Akt may be required for chemoresistance, and that the ability of XIAP to promote cell survival may in part be attributable to its ability to preserve the integrity and function of PI 3-K/Akt pathway by modulating the cellular content of phospho-Akt. Alternatively in the A2780-cp line, cisplatin resistance maybe mediated through a PI 3-K independent mechanism.

The cellular mechanism(s) by which XIAP increases phospho-Akt content in ovarian cancer cells is not known. An increase in phospho-Akt content may be a consequence of changes in activities of upstream kinases and/or phosphatases. Activation of Akt by PI 3-K requires both PtdIns(3,4,5) P3 (the product of PI 3-K) as well as Akt phosphorylation on threonine-308 and serine-473 residues. The binding of PtdIns(3,4,5) P3 to the pleckstrin homology domain of Akt results in its recruitment to the plasma membrane and exposure of its threonine-308 residue. phosphoinositide-dependent kinase has been shown to be responsible for the threonine-308 phosphorylation of Akt (42), although this phosphorylation alone is not sufficient for full Akt activation, which requires PtdIns(3,4,5) P3-dependent serine-473 phosphorylation. Recently, it has been shown that ILK, a serine and threonine protein kinase, may directly phosphorylate Akt on Ser-473 *in vitro* (43). However, the present observations that the PI 3-K inhibitor LY294002 was able to suppress the XIAP-induced phospho-Akt content and to increase apoptosis suggest that the site(s) of regulation by XIAP may either be at PI 3-K or at a step(s) upstream. In this context, because the above-mentioned cellular processes are regulated by the activities of protein tyrosine phosphatases, the possibility exists that XIAP may down-regulate phosphatases known to decrease PI 3-K activity. *PTEN*, a tumor-suppressor gene of a phospholipid phosphatase, is mutated at a high frequency in a wide variety of human gynecological cancers (reviewed in Ref. 44). *PTEN* is capable of dephosphorylating both phosphotyrosine and phosphothreonine, but it also can dephosphorylate the product of PI 3-K (PtdIns(3,4,5)P3, Ref. 45; Ref. 46). ILK activity in *PTEN*-mutant prostate cancer cells is high and can induce Akt phosphorylation, whereas *PTEN* wild type transfection in these cells suppresses ILK activity and Akt activation (47). Because ILK is activated by PtdIns(3,4,5) P3 (43), a mutation in the *PTEN* gene leads to PtdIns(3,4,5) P3 accumulation and ILK activation. Another protein known to decrease PI 3-K activity by directly associating with its p85 subunit is the Src homology 2-containing protein tyrosine phosphatase 1 (48). Whether *PTEN* and/or Src homology 2-containing protein tyrosine phosphatase 1 are involved in the regulation of phospho-Akt levels and chemosensitivity in ovarian cancer cells by XIAP remains to be determined.

Ovarian cancer cells such as OVCAR3 and SKOV3 are more sensitive to LY294002 and undergo apoptosis in the presence of the PI 3-K inhibitor. Because these cancer cell lines have an increased *PI3KCA* [coding for the catalytic  $\alpha$  subunit of PI 3-K (p110)] copy number, this gene is considered an oncogene in ovarian cancer (22). In the present study, we observed high basal levels of phospho-Akt in OVCAR-3, supporting the concept that these cells have an increased PI 3-K activity. This finding may account for the failure of XIAP to increase phospho-Akt content in this cell line, a characteristic not shared by the A2780 and OV2008 cells. Our present results support the importance of PI 3-K/Akt survival pathway in determining the fate of ovarian cancer cells.

In conclusion, our data suggest that the anti-apoptotic action of XIAP may be mediated in part through its interaction with the PI 3-K pathway. A balance in the expression and actions of cell death and

survival factors is critical in cell fate determination (survival *versus* apoptosis). Although the activity of the PI 3-K/Akt survival pathway may be an important contributor to cancer cell chemosensitivity, other pathways (*e.g.*, Fas/FasL) may also be involved (34). The failure of cisplatin to down-regulate XIAP and PI 3-K expression and to induce apoptosis in cisplatin-resistant cells suggests that these survival pathways may be etiologically relevant to chemoresistance. Additional studies are necessary to determine the precise molecular mechanism by which XIAP regulates the PI 3-K/Akt survival pathway.

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