

# Down-Regulation of X-linked Inhibitor of Apoptosis Protein Induces Apoptosis in Chemoresistant Human Ovarian Cancer Cells<sup>1</sup>

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## ABSTRACT

Cisplatin-centered chemotherapy is a key treatment for ovarian cancer, but resistance to chemotherapeutic agents remains a major cause of treatment failure. Multiple factors are known to contribute to the development of this chemoresistance. Although it has been demonstrated that X-linked inhibitor of apoptosis protein (Xiap) prevents apoptosis by inhibiting effector caspases, if and how it is important in chemoresistance in ovarian cancer has not been studied. The effects of Xiap down-regulation and/or restoration of wild type p53 by recombinant adenovirus infection were examined on four ovarian epithelial cancer cell lines [C13\*, A2780-s (wild type p53), A2780-cp (mutant p53), and SKOV3 (null p53)]. Apoptosis and protein expression (e.g., Xiap, caspase-3, p53, MDM2, and p21<sup>waf1</sup>) were assessed by Hoechst 33258 stain and Western blot, respectively. We demonstrated that Xiap down-regulation following adenoviral antisense expression induces apoptosis in the wild-type p53 cells, but not in the mutated or null cells. Xiap down-regulation resulted in caspase-3 activation, caspase-mediated MDM2 processing, and p53 accumulation. Restoration of wild type p53 in the p53-mutated or -null cells significantly enhanced the proapoptotic effect of Xiap antisense expression. Down-regulation of Xiap induced apoptosis in chemoresistant ovarian cancer cells, a process dependent on p53 status.

## INTRODUCTION

Epithelial ovarian cancer is the most lethal gynecological cancer in the Western world and ranks fourth among the most common female cancers. As in other forms of cancer, treatment of ovarian cancer has undergone considerable development during past decades. Chemotherapy and cytoreductive surgery are current standard modalities of treatment for ovarian cancer. Recently, gene therapy (e.g., expression of wild type p53 in patients with p53 mutation) has attracted much attention as a possible mode of treatment for ovarian cancer and is under intensive clinical investigation.

Although platinum derivatives (e.g., cisplatin, carboplatin) and paclitaxel are first-line chemotherapeutic agents for the treatment of ovarian epithelial cancer, chemoresistance is a major therapeutic problem, and the molecular mechanisms involved are poorly understood. The development of resistance may be cell-type specific and related to the dosing schedule (1). The mechanisms of chemoresistance appear to be multifactorial and are generally thought of in terms of altered pharmacodynamics and gene expression (including multi-drug resistant genes), modified drug target, increased rate of DNA repair, or decreased rates of drug-induced DNA or macromolecule damage (2–4). The effect of cisplatin appears to result from inhibition of replication by cisplatin-DNA adducts and G<sub>1</sub> arrest with subse-

quent induction of apoptosis (5). p53 accumulation plays a key role in this cell cycle arrest. It has been shown that the p53 level is regulated by MDM2<sup>4</sup> (6) and that various mutations of p53 are frequently detected in cancer cells. Although our knowledge of the events leading to chemoresistance is incomplete, failure to activate apoptosis in these cancer cells may confer resistance to these agents (4).

An intensive search for modulators of apoptosis has revealed the presence of several potent endogenous suppressors in mammalian cells. The IAP family, originally identified in baculovirus, currently consists of five members: neuronal apoptosis inhibitory protein [Naip], X-linked inhibitor of apoptosis protein [Xiap (8–10)], ilp (10)], human inhibitor of apoptosis protein-1 [Hiap-1 (8, 9, 11)], human inhibitor of apoptosis protein-2 [Hiap-2 (8, 9, 11)], and survivin (12). Hiap 1 and Hiap-2 have been suggested to suppress TNF-receptor signaling by binding to the TNF receptor-associated factor (11), and Xiap suppresses apoptosis via caspase-3 and -7 inhibition (13). It has been reported that IAPs play a central role in the regulation of a number of physiological and pathological processes. Naip is believed to be involved in the pathogenesis of spinal muscular atrophy, in which apoptosis in the spinal ventral neurones is precipitated by the loss of this inhibitor (7). Survivin is highly expressed in various malignant tissues (12, 14) and has been suggested to be an important etiological factor of carcinogenesis and potential new sites for apoptosis-based therapy in cancer (15).

Recent studies from our laboratory have demonstrated that cisplatin is capable of decreasing Xiap protein content in chemosensitive, but not in resistant, hOSE cancer cells, and that over-expression of Xiap in the sensitive cells by adenoviral Xiap sense cDNA delivery prevented the cisplatin-induced apoptosis.<sup>5</sup> These findings suggest that cisplatin-resistance in hOSE cancer may be due to an inability of this chemotherapeutic agent to suppress Xiap protein content and to induce apoptosis.

In the present study, we have examined this possibility by studying the influence of Xiap down-regulation on apoptosis in cisplatin-resistant hOSE cancer cells. Transient infection with adenoviral antisense Xiap cDNA significantly decreased the cellular content of this protein and induced apoptotic cell death as well as enhanced cisplatin-induced cell death in p53 wild-type (C13\*) but not in mutated (A2780-cp) resistant cells. Mechanistic investigations indicate that antisense Xiap-induced apoptosis in the chemoresistant hOSE cancer cells was associated with caspase-3-mediated MDM2 cleavage and increased p53 content. These findings suggested that Xiap is not only regulating down-stream events of caspase-3, but also is triggering p53-dependent pathway when down-regulated by antisense and/or cisplatin treatment.

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<sup>4</sup> The abbreviations used are: MDM2, murine double minute 2; IAP, inhibitor of apoptosis proteins; Xiap, X-linked inhibitor of apoptosis protein; hOSE, human ovarian surface epithelial; DEVD-CHO, N-acetyl-Asp-Glu-Val-Asp-CHO (aldehyde); TBS, Tris-buffered saline; MOI, multiplicity of infection.

<sup>5</sup> J.-L. Li, Q. Feng, J.-M. Kim, D. Schneiderman, P. Liston, M. Li, B. Vanderhyden, W. Fought, M. F. K. Fung, M. Senterman, R. G. Korneluk and B. K. Tsang. Human ovarian cancer and cisplatin resistance: possible role of inhibitor of apoptosis proteins, Endocrinology, in press.

## MATERIALS AND METHODS

**Reagents.** Human polyclonal caspase-3 antibodies and recombinant active caspase-3 were purchased from PharMingen Canada (Mississauga, Ontario, Canada). Horseradish peroxidase-conjugated antimouse and antirabbit antibodies and MDM2 monoclonal antibody for Western blot were obtained from Bio-Rad Canada (Mississauga, Ontario, Canada) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. p53 antimouse monoclonal antibody was obtained from Transduction Laboratories (Lexington, KY). Hoechst 33248 was purchased from Sigma (St. Louis, MO). DEVD-CHO (3) was obtained from BIOMOL Research Laboratories, Inc. Adenoviral wild type sense p53 was provided by Dr. F. L. Graham (McMaster University, Hamilton, Ontario, Canada). Adenoviral LacZ and antisense Xiap constructs, and rabbit polyclonal antihuman Xiap antibody were generous gifts from Dr. R. 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 pGEX vector (Amersham Pharmacia Biotech, Arlington Heights, IL) containing full-length Xiap cDNA. The antibody was affinity-purified by passing through a glutathione S-transferase-Xiap glutathione-Sepharose column. Specificity was confirmed on Western blots (using the antibody-depleted elute from the affinity column), and cross-reactivity with other IAPs was not noted. Construction of recombinant adenovirus and its delivery was carried out as described previously (16). Briefly, the open reading frame of Xiap was PCR-amplified, cloned in the pCR2.1 vector (Invitrogen, Carlsbad CA), and sequenced. The open reading frame was cut out and ligated into the Swa-1 site of pAdex1CAwt cosmid DNA. The vector was packaged with the Promega cosmid packaging extracts and used to infect *E. coli*. Colonies were picked and screened for the presence of the insert in the antisense orientation relative to the chicken  $\beta$ -actin promoter. CsCl purified cosmid DNA was cotransfected with wild-type adenovirus DNA that was allowed to generate infectious adenovirus DNA only when homologous recombination with cosmid DNA occurred. The final recombinant adenovirus contained a linear, double-stranded genome of 44,820 bp plus the antisense Xiap insert (~1,500 bp). Adenoviral expression system was generated with an Ad E1 insertion vector. Virus titer was determined by the plaque assay.

**Cell Culture.** Cisplatin-sensitive (A2780-s), -resistant (C13\*, A2780-cp, and SKOV3) human ovarian epithelial cancer cell lines were gifts from Drs. R. Goal and B. Vanderhyden (Ottawa Regional Cancer Center, Ottawa, Ontario, Canada) and were maintained at 37°C and 5% CO<sub>2</sub>, 95% atmosphere in RPMI 1640 medium (C13\*), DMEM/F12 (A2780-s and A2780-cp), or McCoy 5A (SKOV3; Life Technologies, Inc./BRL, Burlington, Ontario, Canada) supplemented with fetal bovine serum (10%), streptomycin (50  $\mu$ g/ml), penicillin (50 units/ml), and Fungizone (0.625  $\mu$ g/ml; Life Technologies, Inc./BRL). These cell lines were established from a patient with serous cystadenocarcinoma and have been well-characterized in terms of their cisplatin sensitivity (17, 18). C13\* and A2780-cp cells carry wild type p53 and mutant p53, respectively (19, 20). SKOV3 is a null p53 cell line (21). Cells, plated in  $5 \times 10^4$  cells/cm<sup>2</sup>, were treated with adenoviral expression vectors (see below) or freshly prepared *cis*-Platinum (II) Diammine Dichloride [cisplatin 0–100  $\mu$ M (Sigma)] under serum-free conditions. Serum withdrawal had no significant effect on cell viability during the experimental period, as confirmed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

**Hoechst 33248 Staining.** At the end of the culture period, cells attached to the growth surface were removed by trypsin treatment [trypsin (0.05%), EDTA (0.53 mM); 37°C, 1 min]. Attached and detached cells were pooled, pelleted, and resuspended in neutral-buffered formalin (10%) containing Hoechst 33248 dye (12.5 ng/ml). Cells were spotted onto slides and assessed for typical apoptotic nuclear morphology (nuclear shrinkage, condensation, and fragmentation) with Hoechst dye under a Zeiss fluorescence microscope with appropriate filter combination. At least 200 cells/treatment group were counted and assessed randomly selected fields and blinded slides to avoid experimental bias.

**Protein Extraction and Immunodetection.** Cells were pelleted and lysed in ice-cold lysis buffer [NaCl (137 mM), Na<sub>2</sub>HPO<sub>4</sub> (8.1 mM), KCl (2.68 mM), KH<sub>2</sub>PO<sub>4</sub> (1.47 mM), NP40 (1%; v/v), sodium deoxycholate (0.05%; w/v), SDS (0.1%; w/v) containing phenylmethylsulfonyl fluoride (10  $\mu$ M), aprotinin (57  $\mu$ g/ml), leupeptin (5  $\mu$ g/ml), and pepstatin (5  $\mu$ g/ml)]. Cell lysates were sonicated briefly (5 s/cycle, 3 cycles; 0°C), incubated on ice (1 h), and centrifuged (15,000  $\times$  g; 20 min). The supernatant was saved and stored at

–20°C for subsequent analyses. Protein concentration was determined using Bio-Rad DC protein assay kit. Equal amounts of proteins (70–100  $\mu$ g) were loaded and resolved on SDS-PAGE (8–15%) and electroblotted (30V; 12 h) onto nitrocellulose membranes (Bio-Rad Canada). After Western transfer, uniform protein loading was confirmed by comparing the intensity of stained total proteins between lanes of nitrocellulose membranes previously stained with SYPRO Ruby protein stain (Molecular Probes, Eugene, OR). The images were scanned and stored electronically, and the intensity of the protein stain in each lane was quantified for future reference or for normalization of Western blot results. Membranes were then blocked (room temperature, 1 h) with 5% Blotto [Tris-HCl (10 mM; pH 8.0), NaCl (150 mM), Tween 20 (0.05%; v/v); TBS-Tween 20] containing skim milk (5%; w/v), incubated with primary antibody [Xiap (1:4,000); caspase-3 (1:3000); MDM2 (1:1000); p53 (1:2000)]; p21<sup>waf1</sup> (1:2000); room temperature, 1 h] and subsequently with horseradish peroxidase-conjugated secondary antibody [1:2,000 in 5% Blotto; room temperature, 30 min.]. Peroxidase activity was visualized with an ECL kit (Amersham Pharmacia Biotech, Arlington Heights, IL) after a wash with TBS-Tween 20 or TBS [Tris-HCl (10 mM; pH 8.0), NaCl (150 mM)]. Signal intensity was determined densitometrically and normalized against those of total proteins present in the corresponding lane on the membrane using Molecular Analyst software, version 1.5 (Bio-Rad Canada).

**Adenoviral Infection.** After 24 h of plating (10<sup>6</sup> cells/60-mm culture dish), cells were infected with adenoviral antisense full-length Xiap and/or sense p53 at MOIs of 5, 10, 20, or 40 for C13\*, 50, 100, or 200 for A2780-cp; 200 or 400 for SKOV3 (Xiap); and 5, 10, or 20 (p53). Infection with an equivalent concentration (MOI) of LacZ was carried out to provide proper control. LacZ was also used to adjust the total concentration of adenovirus when two different viral preparations were used in the same experiment (double infection). To test the changes on cisplatin sensitivity, infected cells were treated with or without cisplatin (10  $\mu$ M; 24 h) after a 72-h infection period. The number of apoptotic cells was determined by Hoechst stain. Xiap and p53 protein contents as well as caspase-3 and MDM2 contents and cleavage were assessed by Western blot. The infection efficiency over 48 h, as determined by X-gal assay, was >90% in all cell lines, and the expression of Xiap was confirmed, if necessary, by Western blot before cisplatin treatment.

**In Vitro Caspase Treatment.** Whole cell lysate (50  $\mu$ g of total protein) of C13\* cells was incubated with PIPES assay buffer [PIPES (20 mM; pH 7.2), NaCl (100 mM), DTT (10 mM), EDTA (1 mM), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (0.1%; w/v), and sucrose (10%; w/v)] containing recombinant active caspase-3 in the presence or absence of DEVD-CHO. The incubation was terminated by the addition of equal volumes of 2  $\times$  sample buffer [Tris-HCl (100 mM; pH 6.8), DTT (200 mM), SDS (4%; w/v), glycerol (20%; v/v), and Bromphenol blue (0.2%)]. MDM2 cleavage in the cell lysate was assessed by Western blot.

**Statistical Analysis.** All experiments were repeated at least three times. Data were subjected to one- or two-way ANOVA (PRISM software, version 3.0; GraphPad, San Diego, CA). Differences between experimental groups were determined by the Tukey or Bonferroni post-test.

## RESULTS

**Induction of Apoptosis in Cisplatin-resistant Wild Type p53 hOSE Cancer Cells by Adenoviral Xiap Antisense *In Vitro*.** Previous studies have shown that down-regulation of Xiap by Xiap antisense cDNA induced apoptosis in OV2008 cells, a cisplatin-sensitive wild type p53 hOSE cancer cell.<sup>5</sup> In the present study, antisense Xiap delivered by an adenovirus significantly induced apoptosis in its resistant variant, C13\*, which also has the wild type p53 genotype. Without treatment, these cells displayed excellent health and growth characteristics, *i.e.*, polygonal shape with round large nucleus featuring prominent multiple nucleoli and well-spread on the growth surface. Whereas infection of this cell line with an adenovirus carrying LacZ alone (Fig. 1A, *a* and *b*) had no apparent effects on cell morphology, down-regulation of Xiap with adenovirus antisense (MOI = 10; 72 h) evoked typical apoptotic features such as membrane blebbing (*arrow*), cell shrinkage and detachment (Fig. 1A, *c*) and nuclear condensation and fragmentation (Fig. 1A, *d*).

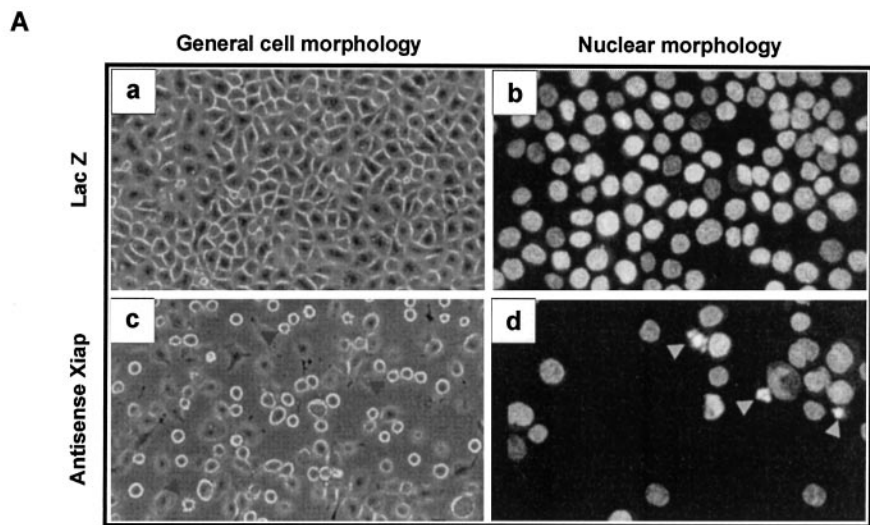
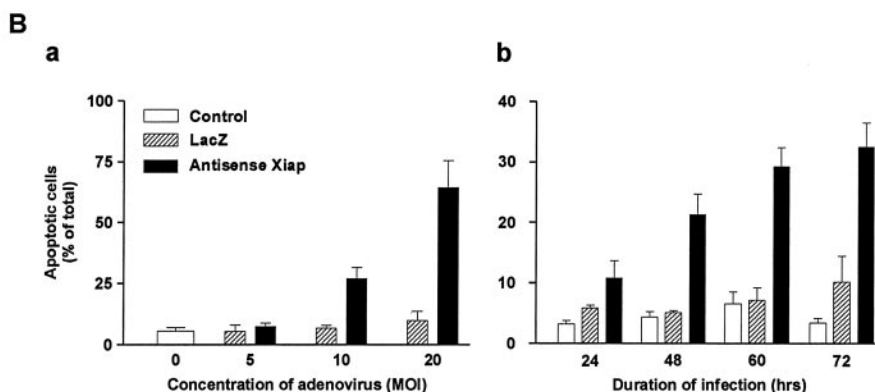


Fig. 1. Influence of adenoviral antisense Xiap on C13\* cells. A, C13\* cells were infected (MOI = 20) with adenoviral LacZ (a and b) or antisense Xiap (c and d). Representative pictures of phase-contrast (general morphology; a and c,  $\times 100$ ) and fluorescent (nuclear morphology; b and d,  $\times 200$ ) images are shown. B, C13\* cells were infected with various concentrations of adenoviral LacZ (▨) or antisense Xiap (■) (MOI = 0, 5, 10, or 20; 72 h; panel a), or for various durations (24, 48, 60, or 72 h; MOI = 20, panel b).



The proapoptotic effect of adenoviral antisense Xiap infection on C13\* cells was concentration (MOI)- and time-dependent. Compared with nontreated control, adenoviral LacZ infection (MOI = 5–20) had no significant effect on apoptosis. However, antisense Xiap significantly increased the number of apoptotic cells at MOIs of 10 ( $27.0 \pm 4.6\%$ ;  $P < 0.05$ ) and 20 ( $64.3 \pm 10.9\%$ ;  $P < 0.001$ ) 72 h after adenoviral infection when compared with equivalent concentrations of LacZ ( $6.8 \pm 1.2\%$  and  $9.9 \pm 3.8\%$ , respectively). Two-way ANOVA indicates that the effects of antisense Xiap ( $P < 0.001$ ) and concentration ( $P < 0.001$ ) were statistically significant, as was the interaction between the two factors ( $P < 0.0001$ ; Fig. 1B, a). Although antisense Xiap infection (MOI = 10) resulted in a statistically nonsignificant ( $10.9 \pm 2.8\%$  versus  $5.9 \pm 0.5\%$ ;  $P > 0.05$ ) increase in the number of apoptotic cells at 24 h, significant and much larger increases were noted at 48 h ( $21.3 \pm 3.4\%$  versus  $5.2 \pm 0.3\%$ ;  $P < 0.01$ ), 60 h ( $29.3 \pm 3.2\%$  versus  $7.1 \pm 2.1\%$ ;  $P < 0.001$ ) and 72 h ( $32.6 \pm 4.0\%$  versus  $10.2 \pm 4.3\%$ ;  $P < 0.001$ ) when compared with LacZ controls (Fig. 1B, b).

**Xiap Down-Regulation Sensitizes Cisplatin-resistant C13\* Cells to the Cytotoxic Action of Cisplatin.** Whereas C13\* cells failed to respond to cisplatin at concentrations as high as  $30 \mu\text{M}$ , a higher concentration ( $100 \mu\text{M}$ ) was capable of inducing apoptosis in  $16.4 \pm 3.9\%$  and  $16.6 \pm 3.0\%$  of these cells. Antisense Xiap treatment sensitized the resistant cells to the proapoptotic action of cisplatin. The number of apoptotic cells in the antisense Xiap treatment group was significantly higher than the LacZ group at 30 ( $14.7 \pm 3.5\%$ ;  $P < 0.05$ ) and 100 ( $48.7 \pm 4.9\%$ ;  $P < 0.001$ )  $\mu\text{M}$  cisplatin and the cisplatin concentration-response curve was shifted to the left by antisense Xiap expression (Fig. 2A). Significant effects of

antisense Xiap ( $P < 0.001$ ) and cisplatin concentration ( $P < 0.001$ ) as well as their interaction ( $P < 0.001$ ) were confirmed by two-way ANOVA. The effect of antisense Xiap was concentration-dependent, as demonstrated in studies with C13\* cells infected with various concentrations (MOI = 0–40) of LacZ or antisense Xiap and then challenged with cisplatin ( $10 \mu\text{M}$ ; 24 h). Antisense Xiap-treated cells showed increased sensitivity to cisplatin at MOIs of 20 ( $P < 0.05$ ) and 40 ( $P < 0.001$ ) compared with LacZ-treated cells. Two-way ANOVA indicates significant antisense ( $P < 0.001$ ) and concentration ( $P < 0.001$ ) effects and interaction ( $P < 0.001$ ; Fig. 2B).

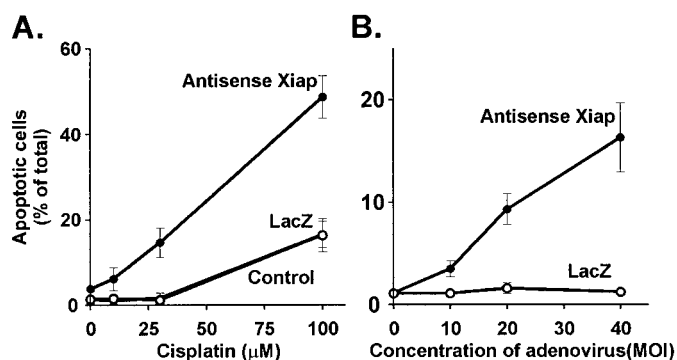


Fig. 2. Adenoviral antisense Xiap infection sensitizes C13\* to cisplatin. A, C13\* cells infected with adenoviral antisense Xiap (●) or LacZ (○) (MOI = 20; 72 h) or without adenovirus (control, ▲) were treated with various concentrations of cisplatin (0, 10, 30, and  $100 \mu\text{M}$ ; 24 h). B, C13\* cells infected with various concentrations of adenoviral antisense Xiap (●) or LacZ (○) (MOI = 0, 10, 20, and 40; 72 h) were treated with cisplatin ( $10 \mu\text{M}$ ; 24 h). Results represent means  $\pm$  SE of three independent experiments.

To confirm the effectiveness of adenoviral Xiap antisense in Xiap down-regulation, extracts from C13\* cells previously infected with adenoviral LacZ or antisense Xiap were subjected to Western analysis 72 h after infection. Whereas Xiap protein content in C13\* cells was not significantly affected by LacZ ( $P > 0.05$ ), it was significantly decreased by antisense Xiap (approximately 40% at MOI = 20;  $P < 0.05$ ). Two-way ANOVA indicates significant antisense Xiap ( $P < 0.05$ ) and concentration ( $P < 0.05$ ) effects (Fig. 3A, a and b). Xiap down-regulation was associated with cleavage of procaspase-3, a 32-kDa member of the cysteine protease family involved in the execution of apoptosis and activated by proteolytic cleavage to form 20- and 18-kDa fragments (prodomain + large subunit and large subunit alone, respectively) by the other upstream caspases (22). The cleavage of procaspase-3 was dependent on the antisense concentration, and significant increases of p20 and p17 protein contents were observable at MOIs of 10 ( $P < 0.001$ ) and 20 ( $P < 0.001$ ; Fig. 3A). Significant antisense ( $P < 0.001$ ) and concentration ( $P < 0.001$ ) effects as well as interaction between these factors ( $P < 0.001$ ) were noted. Consistent with the observed onset of apoptosis, p20 ( $P < 0.01$ ) and p17 ( $P < 0.05$ ) levels were significantly increased (compared with LacZ) as early as 48 h after adenoviral Xiap antisense infection (Fig. 3B, a and b). A significant interaction ( $P < 0.05$ ) existed between antisense Xiap and the duration of infection, which was brought about by the significantly higher levels of these active cleavage fragments after 24 h of infection with the antisense. Interestingly, procaspase-3 levels were decreased, though not consistently, by antisense Xiap treatment.

**Caspase-3-mediated MDM2 Cleavage and p53 Accumulation Associated with Xiap Down-Regulation.** MDM2 is known to play a key regulatory role in p53 stability and is cleaved during apoptosis (23). Whereas the involvement of caspase-3 has been suggested in MDM2 processing (23, 24), the possibility of Xiap being important in

the control of p53 content by maintaining MDM2 integrity (25, 26), remains to be determined. Instead of the 92-kDa intact MDM2 protein previously reported present in high abundance in mammalian cells, a 60-kDa fragment was found to be the dominant isoform in the resistant hOSE cancer cells. Whereas LacZ had no significant influence on the 60-kDa protein content and its cleavage, a decrease of this protein and the appearance of cleaved product (30 kDa) were noted after Xiap down-regulation (Fig. 4A, a). Furthermore, p53 accumulation following Xiap down-regulation at a MOI of 10 ( $P < 0.05$ ) or 20 ( $P < 0.001$ ) was associated with MDM2 cleavage when compared with respective LacZ controls (Fig. 4A, b). The addition of recombinant active caspase-3, but not of caspase-7, to C13\* whole cell lysate elicited a concentration- and time-dependent MDM2 cleavage pattern identical to that observed in extracts from cells following Xiap down-regulation (Fig. 4B, a). MDM2 cleavage by caspase-3 was blocked by the presence of the caspase-3 inhibitor, DEVD-CHO (Fig. 4B, b). Because C13\* cells possess wild type p53 (27), these findings suggest that Xiap down-regulation not only induces caspase-3 cleavage and activation, but possibly also activates a p53-dependent apoptotic pathway.

**p53-dependent Induction of Apoptosis in Cisplatin Resistant hOSE Cancer Cells by Adenoviral Antisense Xiap Expression.** To further examine the involvement of p53 in the Xiap regulated caspase-3 pathway, a pair of human ovarian epithelial adenocarcinoma cell lines (A2780-s and A2780-cp) were also subjected to antisense Xiap expression. In 24-h cultures of A2780-s [a parental, cisplatin-sensitive wild type p53 cell line (19)], cisplatin significantly decreased Xiap content and induced apoptosis in a concentration-dependent manner ( $P < 0.001$ ; Fig. 5A, a and b), whereas it was ineffective in its p53-mutated resistant variant, A2780-cp (Ref. 19;  $P > 0.05$ ; Fig. 5A, c and d). Contrary to the wild type p53 C13\* cells, Xiap down-regulation in A2780-cp alone failed to significantly induce

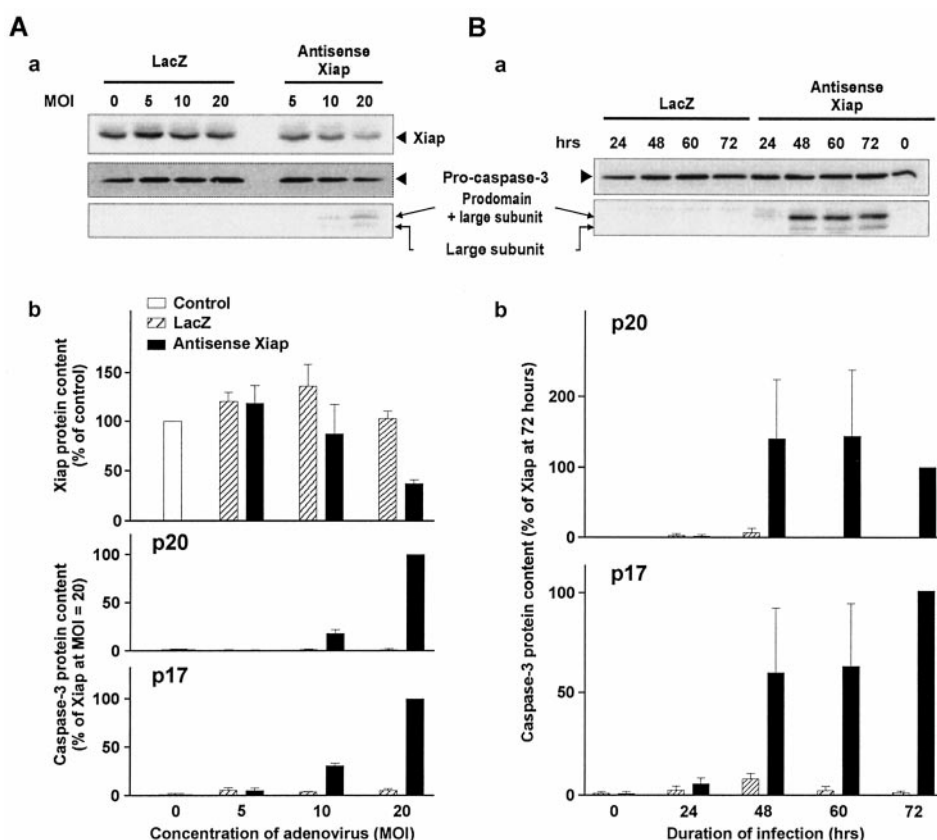
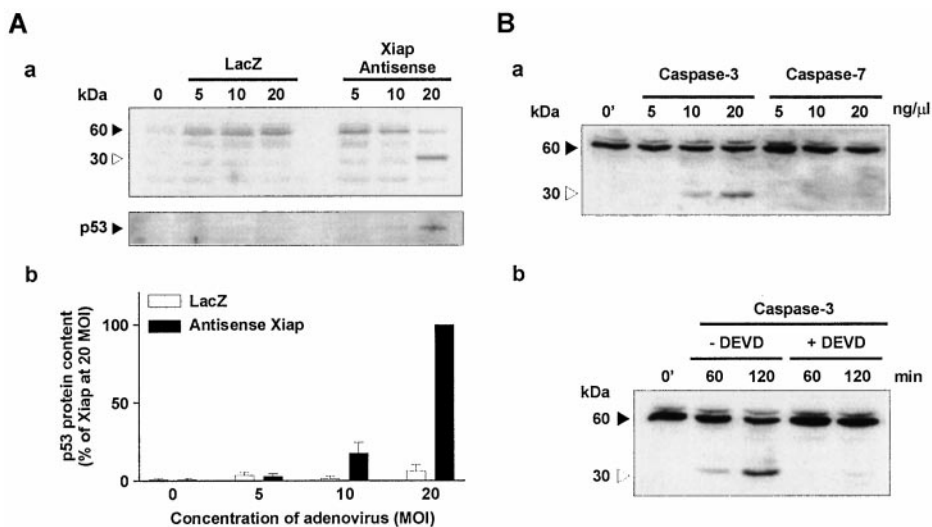


Fig. 3. Influence of Xiap down-regulation on procaspase-3 cleavage in C13\* cells. C13\* cells were infected with adenoviral LacZ (▨) or antisense Xiap (■) (A, MOI = 0, 5, 10, or 20 for 72 h; B, MOI = 10 for 24, 48, 60, or 72 h). Xiap protein content and pro-caspase-3 cleavage were analyzed by Western blot. Representative membranes (a in A and B), and densitometric analysis (b in A and B), are shown. Results represent means ± SE of three independent experiments.

Fig. 4. Influence of Xiap down-regulation on MDM2 cleavage and p53 protein content and caspase-3 on MDM2 processing. **A**, C13\* cells were infected with various concentrations of adenoviral LacZ or Antisense Xiap (MOI = 5, 10, or 20). Representative Western blots for MDM2 cleavage and p53 (**a**) are presented. Densitometric analysis for p53 content are shown on **b**. Results represent means  $\pm$  SE of three independent experiments). **B**, C13\* cell whole-cell lysate was incubated with various concentrations of human recombinant active caspase-3 or caspase-7 (5, 10, and 20 ng/ml) in the absence (**a**) or presence (**b**; 25  $\mu$ M) of DEVD-CHO. Representative Western blots showing MDM2 cleavage in C13\* whole-cell lysate are shown: 60-kDa MDM2 isoform (closed arrow), 30-kDa fragment (open arrow).



apoptosis ( $P > 0.05$ ) even at a concentration of the adenoviral Xiap antisense as high as a MOI of 200, which effectively decreased Xiap content after 72 h (Fig. 6A, *a* and *b*). In contrast, overexpression of wild type p53 alone in these p53 mutants by adenoviral sense cDNA resulted in a concentration (MOI)-dependent increase in apoptosis ( $P < 0.001$ ; Fig. 6A, *c* and *d*). Whereas expression of wild type p53 with low concentration of sense p53 (MOI = 10) had a slight, but nonsignificant, effect on apoptosis ( $P > 0.05$ ), a higher concentration (MOI = 40) markedly increased the population of apoptotic cells ( $25.0 \pm 6.0\%$  versus  $0.8 \pm 0.3\%$ ;  $P < 0.001$ ) compared with corresponding LacZ-treated cells.

Although neither Xiap down-regulation (MOI = 100) nor wild type p53 over-expression (MOI = 10) alone was effective in inducing significant apoptosis [ $5.0 \pm 1.1\%$  (antisense Xiap alone) or  $9.8 \pm 1.9\%$  (sense p53 alone) versus  $2.2 \pm 1.0\%$  (LacZ);  $P > 0.05$ ], coinfection of antisense Xiap and sense p53 led to a marked increase in the number of apoptotic cells ( $35.3 \pm 5.32\%$ ;  $P < 0.01$ ; Fig. 6B). Moreover, the proapoptotic effects of Xiap down-regulation (antisense Xiap; MOI = 100) were dependent on the concentration of sense p53 (Fig. 6C). Expression of antisense Xiap significantly enhanced the sensitivity of the cells to wild type p53 resulting in a shift of the p53 sense concentration-apoptosis response curve to the left. Two-way ANOVA indicates significant antisense Xiap and p53 sense concentration effects, and interaction between the two factors ( $P < 0.01$ ).

Upon wild type p53 restoration (MOI = 10), the proapoptotic effect of antisense Xiap treatment was concentration-dependent ( $P < 0.01$ ), and significant increases in the number of apoptotic cells were evident with adenoviral Xiap antisense at MOIs of 50 ( $37.8 \pm 5.3\%$ ) and 100 ( $51.4 \pm 0.4\%$ ) compared with LacZ ( $7.7 \pm 3.6\%$  and  $11.1 \pm 7.0\%$ , respectively;  $P < 0.01$ ; Fig. 7A, *a*). Adenoviral Xiap antisense expression resulted in a concentration-dependent Xiap down-regulation, however a greater decrease in Xiap level was observed when the A2780-cp cells were coinfecting with Xiap antisense and wild type p53 sense (Fig. 7A, *b* and *c*). Expression of p21<sup>waf1</sup> and 90-kDa MDM2 protein were found predominantly in cells in which wild type p53 was reconstituted. Xiap down-regulation significantly increased p53 content (about three-fold) in p53 mutant reconstituted with wild type p53 and marked by decreased p53-induced p90 and p60 MDM2 levels (approximately 50% and 30%, respectively; Fig. 7A, *d*). These results suggested that caspase-3 activation is necessary, but not sufficient, for the induction of apoptosis in p53-deficient cells, and that a functional p53 may be involved in the completion of the apoptotic process.

To further examine this hypothesis, the effect of antisense Xiap and/or sense p53 delivery on the null p53 cell line (SKOV3) was tested (28). A significant accumulation of p53 protein was detected in the cells treated concurrently with antisense Xiap and sense p53 (Fig. 7B). Induction of intact MDM2 protein was observed in cells with sense p53 expression, irrespective of antisense Xiap delivery, and a caspase-mediated MDM2 fragment was evident in the cells treated with antisense Xiap and sense p53 constructs. Although the proportions of apoptotic cells induced by adenoviral gene delivery were smaller than in C13\* and A2780-cp cell lines, adenoviral infection of cells with antisense Xiap and sense p53 significantly induced apoptosis ( $3.4 \pm 0.39\%$ ) compared with viral LacZ ( $0.6 \pm 0.3\%$ ;  $P < 0.001$ ), antisense Xiap ( $1.7 \pm 0.1\%$ ;  $P < 0.05$ ), and sense p53 ( $0.47 \pm 0.27\%$ ;  $P < 0.001$ ) alone.

## DISCUSSION

It is well established that cisplatin induces DNA damage through formation of inter- and intra-strand cross-linkages and activation of p53-dependent apoptotic pathways. Alterations in cisplatin cellular retention or excretion, DNA repair mechanism, or apoptotic machinery have been considered important etiological factors in chemoresistance. We have demonstrated previously that the addition of cisplatin

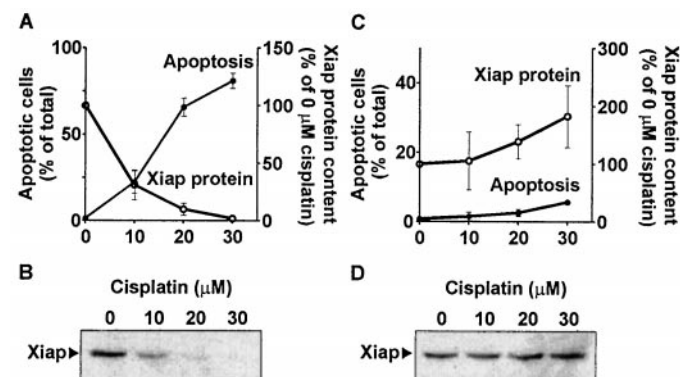


Fig. 5. Influence of cisplatin on Xiap content and apoptosis in cisplatin-sensitive wild type p53 (A2780-s) and -resistant p53 mutated (A2780-cp) hOSE cancer cells. **A**, cells were treated for 24 h with various concentrations of cisplatin (0, 10, 20, and 30  $\mu$ M). Apoptosis (●) and densitometric value of Xiap content (○) in A2780-s (**A**) and A2780-cp (**C**) are shown. **B** and **D**, are representative Western blots for Xiap in A2780-s and A2780-cp cells, respectively.

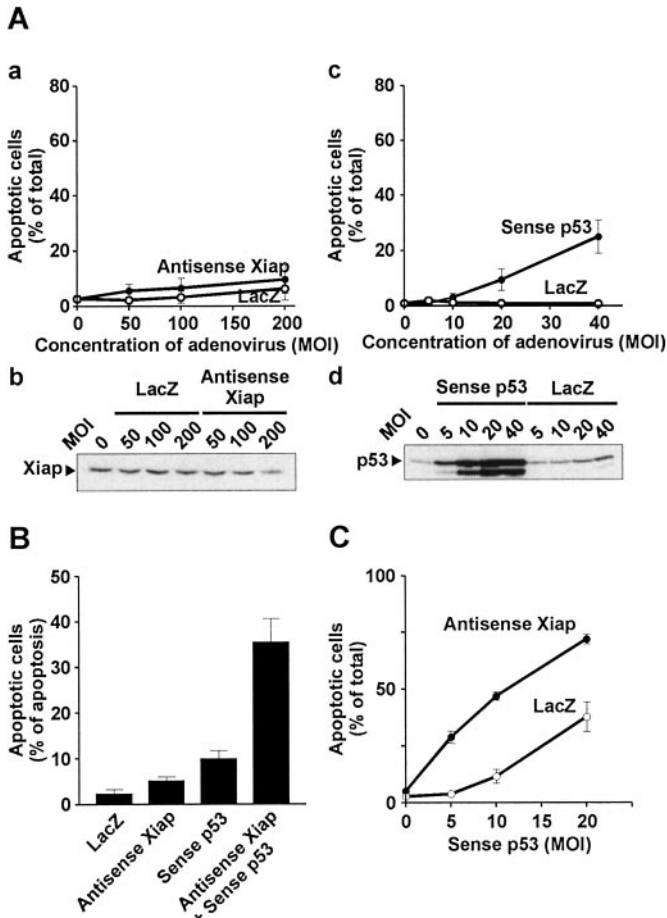


Fig. 6. Influence of Xiap down-regulation and/or wild type p53 restoration on apoptosis in p53 mutated hOSE cancer cells. A, A2780-cp cells were infected with adenoviral LacZ (○) or antisense Xiap (●) [MOI = 0, 50, 100, or 200; 72 h; a and b)] or sense p53 (●; MOI = 0, 5, 10, 20, or 40; 48 h; c and d). Apoptosis is shown in a and c. Panels b and d show representative Western blots for Xiap and p53, respectively. B, synergistic effect of adenoviral wild type p53 sense (MOI = 10) and antisense Xiap (MOI = 100; 72 h) expression on apoptosis. Total concentration of adenovirus was adjusted with LacZ to a final concentration of MOI = 110. C, concentration effects of sense p53 (MOI = 0, 5, 10, or 20) on LacZ, (○) and antisense Xiap (●) (MOI = 100; 72 h) -induced apoptosis in A2780-cp. Results represent means ± SE of three independent experiments.

to hOSE cancer cell cultures decreased Xiap protein content and induced apoptosis in cisplatin-sensitive cells (OV2008 and A2780-s) but not in their respective resistant variants (C13\* and A2780-cp).<sup>5</sup> In addition, over-expression of Xiap in the sensitive cells (OV2008) by adenoviral sense Xiap infection effectively attenuated cisplatin-induced apoptosis, suggesting that Xiap may be an important determinant in cisplatin sensitivity. We hereby have extended these studies to further investigate this concept and have shown that Xiap down-regulation in wild type p53-resistant hOSE cancer cells (C13\*) by antisense Xiap expression sensitizes the cells to cisplatin, with a significant shift of the concentration-apoptotic response curve to the left. Although low cellular cisplatin retention is believed to be an important factor in chemoresistance in this cell line (29), our present findings represent the first demonstration of a possible role of Xiap in chemoresistance in cancer cells and offer a new target for the development of a novel gene therapeutic approach. These results also suggest that cisplatin resistance may be a multifactorial phenomenon.

We have also shown in the present studies that antisense Xiap expression alone in cisplatin-resistant wild type p53 hOSE cancer cells (C13\*) results in a concentration- and time-dependent decrease in Xiap content and an increase in apoptosis. These findings not only demonstrate a key regulatory role of Xiap in apoptosis in these cells,

but also suggest that, despite being resistant to cisplatin, they are capable of and committed to apoptosis but are prevented from undergoing the death process due to the presence of Xiap. Xiap has a strong affinity for caspase-3, a cysteine protease involved in the execution of apoptosis (13, 30) and is an inhibitor of caspase-3 activation (cleavage by upstream caspases) and activity. The physiological role of Xiap has been considered as a final guardian for preventing and regulating this cell-death process. Thus, removal of the antiapoptotic factor would be expected to have resulted in unleashing of the apoptotic process, probably mediated by the release of caspase-3 from its inhibition. However, our present observations are that Xiap down-regulation alone was only effective in p53 wild type cells, and failed to induce apoptosis in p53 mutated and null cell lines.

The mechanism(s) by which caspase-3 elicits its proapoptotic ac-

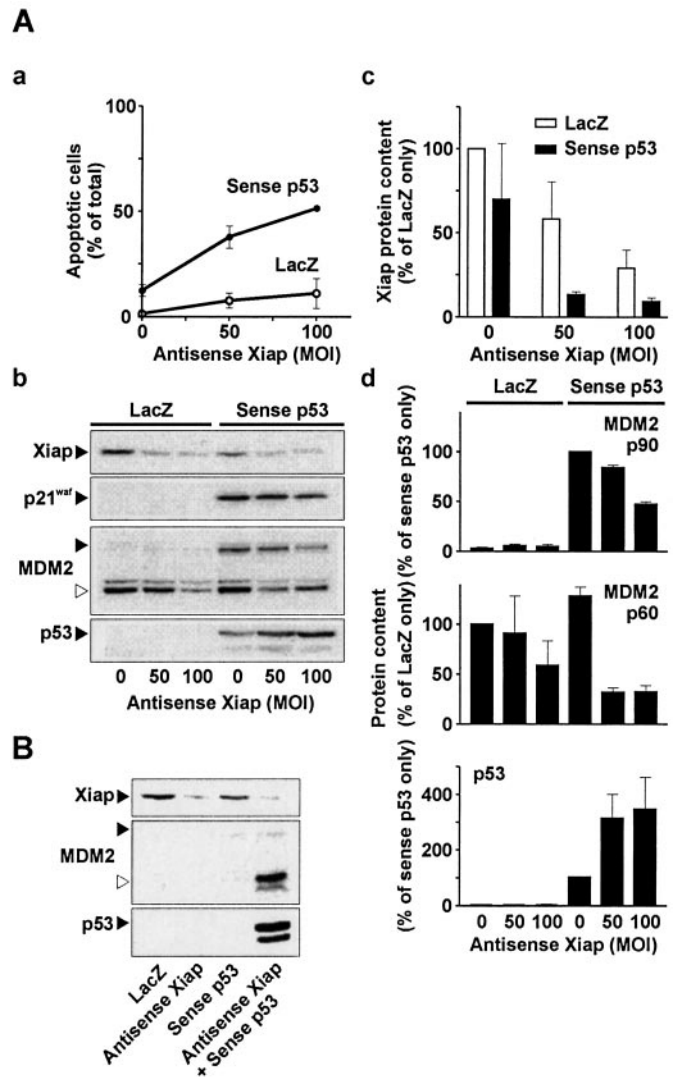


Fig. 7. The effect of Xiap down-regulation and wild type p53 restoration, alone or combined on MDM2 processing, p53/p21<sup>waf1</sup> contents, and apoptosis in hOSE cancer cell lines. A, A2780-cp cells were infected with various concentrations of adenoviral antisense Xiap (MOI = 0, 50, or 100; 72 h), alone or with adenoviral LacZ or p53 sense (MOI = 10). a, apoptosis [LacZ (○); sense p53 (●)]. b, representative Western blots showing Xiap, p21<sup>waf1</sup>, MDM2 cleavage [90-kDa intact form (closed arrow); 60-kDa isoform (open arrow)], and p53 contents. Densitometric analysis of changes in Xiap [c, LacZ (□) and sense p53 (■) expression], MDM2 and p53 (d) contents are shown. Results represent means ± SE of three independent experiments. B, SKOV3 cells were infected with adenoviral wild type p53 sense (MOI = 10) and/or antisense Xiap (MOI = 400; 72 h). Total concentration of adenovirus was adjusted with LacZ to a final concentration of MOI = 410. Representative Western blots for Xiap, MDM2, and p53 are shown. Open arrow indicates cleaved MDM2.

tion in hOSE cancer cells is unclear. It has been suggested that proteins involved in DNA breakdown [*e.g.*, inhibitor of caspase activated DNase (31)] and signal transduction [*e.g.*, focal adhesion kinase, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (32)] and cytoskeletal proteins [*e.g.*, actin (33)] are possible physiological substrates for caspase-3, and that this death protease mediates the apoptotic signal by disrupting survival signals and/or cellular integrity. In the present studies, we have observed that pro-caspase-3 cleavage following Xiap down-regulation in wild type p53 hOSE cancer cells was temporally associated with MDM2 cleavage. In both C13\* and A2780-cp cells, the 60-kDa MDM2 was the dominant isoform. MDM2 were undetectable in SKOV3 cells, although a 90-kDa native protein became detectable after wild type p53 over-expression. Whereas the 60-kDa isoform is believed to be a caspase cleavage product of the 90-kDa protein and is frequently found in cancer cells (34), our *in vitro* studies with C13\* whole-cell lysate and human recombinant active caspase-3 and -7 indicate that MDM2 may be a physiological substrate for caspase-3. We demonstrated the caspase-3-mediated cleavage of the 60-kDa MDM2 to a smaller fragment (30 kDa), as well as the 90- to 60-kDa cleavage when the 90-kDa protein was up-regulated. Although how the conversion of the 60-kDa protein to the 30-kDa form (but not of the 90- to 60-kDa form) is suppressed in the nonapoptotic cells is unclear, the possible involvement of Xiap-independent caspases in the initial processing of the 90-kDa protein cannot be excluded. These findings are, however, consistent with the fact that MDM2 has caspase-sensitive sites (23), and are in good agreement with previously published observations (24, 35). The physiological significance of the observed caspase-3 specificity is not known.

MDM2 is an oncoprotein that binds p53 and facilitates ubiquitin-mediated degradation of the tumor suppressor protein (6). It has been reported that a decrease in MDM2 content stabilizes p53, whereas the opposite is true when the oncoprotein is over-expressed (36). Wild type p53 accumulation is known to induce cell cycle arrest and apoptosis, and p53 mutation is frequently identified in various cancer cells (37). In the present investigations, we have demonstrated Xiap down-regulation is associated with significant cleavage of caspase-3 and MDM2, p53 accumulation, and increased apoptosis in wild type p53-expressing C13\* cells. When wild type p53 was supplemented, accumulation of p53 was also evident in p53 mutated (A2780-cp) and null (SKOV3) cells treated with antisense Xiap. Xiap down-regulation together with wild type p53 reconstitution (by sense p53 expression), however, not only further increased p53 content, but significantly increased apoptosis in these otherwise p53-deficient cells. These studies provide the first evidence for a role of Xiap in the regulation of cellular p53 level and suggest that caspase-3-mediated MDM2 processing may be an additional regulatory point for the antiapoptotic protein in promoting survival of wild type p53-expressing hOSE cancer cells.

The regulation of Xiap levels in hOSE cancer cells remains to be investigated. In the present studies, over-expression of wild type p53 decreased basal Xiap content and enhanced the effect of antisense Xiap expression, suggesting a possible involvement of p53 in the transcriptional control of Xiap expression. On the other hand, posttranscriptional processing of Xiap may also be an important determinant of the steady-state levels of Xiap and, thus, of apoptosis. Our present studies indicate that at least 60–72 h are required to sufficiently decrease Xiap content following antisense expression, suggesting that the antiapoptotic protein may have a relatively long half-life. This is consistent with the observation that, although adenoviral infection efficiency was close to 100%, only about 60% of the cells were apoptotic 72 h after infection (MOI = 20).

In summary, we have shown that Xiap plays an important role of regulation of apoptosis in hOSE cancer cells and is an important determinant in cisplatin resistance. In addition to its well-established inhibitory action on caspase-3 activity, this antiapoptotic protein appears to be central in the control of p53 accumulation and p53-mediated apoptosis, possibly via the MDM2-p53-ubiquitin degradation pathway. Xiap down-regulation not only induces apoptosis but also sensitizes chemoresistant wild type p53 hOSE cancer cells to the proapoptotic action of cisplatin. In p53 mutant cancer cells, coexpression of Xiap antisense and wild type p53 sense is more effective than wild type p53 restoration alone. These findings provide a new concept for the development of novel therapeutic approaches in the treatment of chemoresistant hOSE cancer. Studies with xenograft models are required to establish whether these strategies are indeed applicable *in vivo*.

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## Down-Regulation of X-linked Inhibitor of Apoptosis Protein Induces Apoptosis in Chemoresistant Human Ovarian Cancer Cells

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