Cisplatin Induces p53-Dependent FLICE-Like Inhibitory Protein Ubiquitination in Ovarian Cancer Cells

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Abstract

Understanding the mechanism of cisplatin (CDDP) action may improve therapeutic strategy for ovarian cancer. Although p53 and FLICE-like inhibitory protein (FLIP) are determinants of CDDP sensitivity in ovarian cancer, the interaction between p53 and FLIP remains poorly understood. Here, using two chemosensitive ovarian cancer cell lines and various molecular and cellular approaches, we show that CDDP induces p53-dependent FLIP ubiquitination and degradation, and apoptosis in vitro. Moreover, we showed that Itch (an E3 ligase) forms a complex with FLIP and p53 upon CDDP treatment. These results suggest that p53 facilitates FLIP down-regulation by CDDP-induced FLIP ubiquitination and proteasomal degradation. [Cancer Res 2008;68(12):4511–7]

Introduction

Cisplatin (CDDP) is the first-line anticancer agent for human ovarian cancer and is known to act in part by induction of apoptosis (1). In CDDP-sensitive cells, it also decreases cellular levels of FLICE-like inhibitory protein (FLIP), a FADD-binding suppressor of apoptosis, which is present as two splice variants: FLIPL (55 kDa) and FLIPS (28 kDa). FLIP contains two NH2-terminal death effector domains (DED), which prevent caspase-8 activation through DED-DED interaction (2). Although FLIP down-regulation is an important factor in CDDP-mediated apoptosis, its mechanism remained unclear (3).

The ubiquitin-proteasome pathway (UPP) could be involved in regulating FLIP. UPP is a major regulatory mechanism for intracellular protein level. This process is mediated by an E1 (Ub activating enzyme), E2 (Ub conjugase), and E3 (Ub ligase) complex. Typically, proteins modified by polyubiquitin chains are recognized and degraded by the proteasome (4). Importantly, Itch, a member of the HECT family of E3 ligases, interacts with FLIP and is believed to mediate its degradation as well as tumor necrosis factor α–induced apoptosis (5).

p53 is a transcription factor that regulates cell cycle progression, DNA repair, and apoptosis. p53 is maintained at low levels by its negative regulator, MDM2, which ubiquitinates p53, targeting it for proteasomal degradation (6). TP53 mutations are frequently observed in human ovarian cancer cells (7) and associated with decreased chemoresponsiveness (8).

In the present study, we investigated the involvement of Itch and p53 in CDDP-induced FLIP down-regulation. We showed that CDDP enhances FLIP-p53-Itch interaction, inducing FLIP ubiquitination and degradation in a p53- and Itch-dependent manner. These results suggest that the modulation of FLIP content may be an effective strategy to overcome chemoresistance in ovarian cancer.

Materials and Methods

Reagents. MG132, Lactacystin, and Epoxomicin were from Calbiochem (Ab-1). Cell Signaling, Inc., Ambion, and Dharmacon, Inc. provided siRNA for p53, Itch, and control, respectively. Ribonucleic and Lipofectamine Plus were from Novagen and Invitrogen, respectively. HA-tagged ubiquitin was provided by Dr. Qiao Li (University of Ottawa, Canada). Adenoviral cDNAs were synthesized at the University of Ottawa Neuroscience Research Institute. Primary antibodies for immunoblot were mouse monoclonal anti-p53 (DO-1; Santa Cruz Biotechnologies), anti-FLIP (NF6; Alexis; Ab-1) and anti-Itch (BD Bioscience), anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ab8245; Abcam), and anti-V5 (Invitrogen). Antibodies for precipitation were goat polyclonal anti-HA and anti-V5 (Bethyl Laboratories). Anti-pan-histone (H3) and anti-p53 (DO-1; Santa Cruz Biotechnologies), and for immunofluorescence were rabbit polyclonal anti-FLIP antibody (Cell Signaling Technology) and anti-p53 (C-19; Santa Cruz Biotechnologies), and mouse monoclonal anti-Itch (BD Bioscience). Donkey secondary antibodies were from Jackson Immunoresearch.

Cell culture and adenovirus infection. Chemosensitive ovarian cancer cells (OV2008 and A2780) were cultured as previously reported (8). Cells were infected with appropriate adenoviral constructs as indicated in the text. LacZ adenovirus was used to normalize each treatment group to total adenoviral concentration. Adenovirus infection efficiency [multiplicity of infection (MOI), 5; 24 h] was >90% (8).

FLIP ubiquitination analysis. Cells were transfected with HA-ubiquitin. (3). After 24 h, spent medium was replaced with fresh RPMI 1640 or DMEM F12 containing CDDP (0–10 μM; 0–9 h) and Epoxomicin. Cells were harvested for FLIP ubiquitination analyses (9). The cell pellet was resuspended in boiling 1% SDS in PBS, heated (100°C for 5 min), and suspended in 1% Triton X-100 in PBS (1:10). DNA was sheared by sonication, and after centrifugation (14,000 × 15 g for 15 min), the supernatant [diluted with 1% Triton X-100 and 0.5% bovine serum albumin in PBS (1:1)] was incubated (overnight for 4°C with primary antibody. The beads were washed (6 times with 1% Triton X-100 in PBS), and the precipitated proteins were immunoblotted.

RNA interference. Cells transfected for 24 h with p53 siRNA (100 nmol/L), Itch siRNA (100 nmol/L), or control siRNA (100 nmol/L; ref. 3) were treated with CDDP and harvested for subsequent analysis.

Western blotting. Western blotting (WB) was done as previously described (3). Membranes were incubated with anti-FLIP (NF60; 1:500), anti–GAPDH (1:20,000), anti-p53 (1:1,000), anti-Itch (1:500), or anti-V5 (1:5,000; overnight for 4°C), and with horseradish peroxidase–conjugated anti-rabbit or anti-mouse secondary antibody (1:1,000–10,000; 1 h at room temperature). Peroxidase activity was visualized with the enhanced chemiluminescent kit (Amersham Biosciences) and analyzed (Scion Image software; Scion, Inc.).
**FLIP mRNA analysis.** Relative differences in FLIP mRNA levels in experimental groups were determined semiquantitatively by reverse transcription-PCR (RT-PCR). Total RNA was reverse transcribed followed by PCR (10).

**Assessment of apoptosis.** Apoptosis was determined morphologically, using Hoechst 33258 nuclear stain (3). The counter was "blinded" to avoid experimental bias.

**Immunoprecipitation.** Immunoprecipitation IP was performed on whole cell lysates using anti-V5 and -p53 antibodies (11) and immunoblotted for p53, Itch, and V5.

**Immunocytochemistry and confocal microscopy.** Cultured OV2008 cells were fixed with methanol (10 min at −20°C). Nonspecific binding was blocked with 0.8% (w/v) serum albumin and 1% gelatin in PBS (30 min at room temperature; ref. 12). Cells were then incubated with anti-p53 (1:50), anti-FLIP (1:25), and anti-Itch (1:25; overnight at 4°C) and subsequently with secondary donkey-conjugated antibodies: anti-goat (Cy5), anti-mouse (Cy3), and anti-rabbit (FITC; 1:25; 2 h at room temperature). Cells were mounted with Vectashield (Vector). Cells incubated without primary antibodies served as negative controls. Fluorescence images (1,024 × 1,024 pixels) were acquired using a LSM 510 confocal laser-scanning microscope.

![Figure 1](https://example.com/image1.png)

Figure 1. CDDP down-regulates FLIP through Itch-dependent proteasomal degradation. A, CDDP decreased FLIPs and FLIPS contents and induced apoptosis in a concentration-dependent manner in OV2008 and A2780s cells (***, P < 0.001 versus control (CTL); n = 3). OV2008 and A2780s cells were cultured with CDDP (0–10 μmol/L; 24 h) and assessed for FLIP and GAPDH contents by WB (top), and for apoptosis by Hoechst 33258 nuclear staining (bottom). GAPDH served as a protein loading control. B, CDDP, at concentration that decreased FLIP protein contents (10 μmol/L), failed to elicit a significant influence on FLIP mRNA abundance (P > 0.05, n = 3; top). OV2008 and A2780s cells were cultured for different duration (0–24 h) with CDDP (0–10 μmol/L). FLIP mRNA abundance was determined by RT-PCR (n = 3); WB analysis indicating that the CDDP-induced FLIPs and FLIPS down-regulation was attenuated by the presence of proteasome inhibitors (***, P < 0.001; n = 3; bottom). OV2008 cells were pretreated with the proteasome inhibitors MG132 (5 μmol/L), Lactacystin (10 μmol/L), and Epoxomicin (25 nmol/L) and then treated for 12 h with CDDP or DMSO (control). C to D, CDDP-induced FLIP ubiquitination is dependent on Itch (n = 3). OV2008 and A2780s transfected with HA-ubiquitin (2 μg; 24 h) or infected (MOI, 25; 24 h) with adenoviral V5-FLIPs (V5-FS; C), V5-FLIPS (V5-FS; D), or LacZ (as control) in the absence or presence of Itch siRNA (100 nmol/L; 24 h); and then treated with CDDP (0–10 μmol/L) and Epoxomicin (25 nmol/L) to prevent proteasomal degradation of ubiquitinated FLIP. Cells only infected with LacZ (lane 2) or transfected with HA-ub (lane 3) are indicated. At the end of 1.5 and 3 h, cells were harvested for assessment of Itch and GAPDH contents (C and D, top; WB) as well as V5-FLIPS and V5-FLIPS ubiquitination (C and D, bottom, respectively; IP (anti-HA-ubiquitin, IgG as control) and WB (V5-FS or V5-FS)).
Results and Discussion

CDDP down-regulates FLIP protein content via FLIP proteasomal degradation. Our recent study shows that CDDP down-regulates FLIP content and induces apoptosis in ovarian cancer cells (3). To better understand how CDDP decreases FLIP level in ovarian cancer cells, two ovarian cancer cell lines (OV2008 and A2780s) were cultured with CDDP (0–10 μmol/L; 24 hours) and

Figure 2. CDDP enhances p53-FLIP-Itch interaction and FLIP ubiquitination. FLIP-p53-Itch interaction and FLIP ubiquitination were enhanced by CDDP (lane 5) after 1.5 h for FLIP S (C, top) and 3 h for FLIP L (A, top). OV2008 and A2780s transfected with HA-ubiquitin (HA-ub; 2 μg; 24 h), subsequently infected (MOI, 25; 24 h) with either adenoviral V5-FLIP S (A–B), V5-FLIP L (C–D), or LacZ (as control) and cultured for different duration with CDDP (0–10 μmol/L) and Epoxomicin (25 nmol/L). Cells only transfected with HA-ubiquitin (lane 1) or infected with adenoviral LacZ (lane 3) are indicated. Protein-protein interaction was determined by IP-WB. p53, FLIP, and ubiquitin immunoprecipitates were immunoblotted (IP, p53; WB, V5 and Itch; IP, V5-tagged FLIP S; WB, p53 and Itch (A and C); IP, HA-tagged ubiquitin (B and D); WB, V5-FLIP, or FLIP S (n = 3)).
FLIP content was determined. CDDP decreases FLIP<sub>L</sub> and FLIP<sub>S</sub> levels in a concentration-dependent manner (Fig. 1A). FLIP down-regulation was associated with increased apoptosis (Fig. 1A). The down-regulation of FLIP by CDDP (0–10 μmol/L; 0–24 hours) does not seem to be associated with any change in FLIP<sub>L</sub> and FLIP<sub>S</sub> mRNA abundance (Fig. 1B, top).

To determine whether proteasomal degradation could be involved in CDDP-induced FLIP down-regulation, OV2008 cells...
Figure 4. CDDP induces FLIP-p53-Itch triple colocalization at OV2008 cell membrane. Itch-, FLIP-, and p53-IR displayed both clusters and diffuse staining in control cells (A). Clusters were restricted to the cytoplasm and rarely colocalized (arrowhead). In contrast, CDDP treatment (C) resulted in Itch-, FLIP-, and p53 clusters at the cell membrane, which were mostly colocalized (arrowheads). Fluorescence intensity profiles (B and D) obtained from OV2008 cell membrane magnifications of A8 (control) and C8 (CDDP treated) are representatives of diffuse and clustered expression pattern, respectively for Itch, FLIP, and p53. Although diffuse pattern is represented by constant fluorescence intensity along the membrane of control cells (B1–4), clustered pattern is indicated by hotspots of fluorescence in CDDP-treated cells (D1–4), which also exhibited similar Itch, FLIP, and p53 fluorescence profiles. OV2008 cells were treated with DMSO (control, A and B) or CDDP (0–2.5 μmol/L, 2 h; C and D). Protein-protein colocalization was assessed by immunocytochemistry and confocal microscopy. A and C, immunofluorescent confocal sections of OV2008 cells treated with DMSO or CDDP and triple labeled for Itch (A1 and C1), FLIP (A2 and C2), p53 (A3 and C3), and merged images (A5–8 and C5–8). White arrow on A8 and C8 indicates the direction of intensity profile measurement (B1–4 and D1–4, respectively). Scale bars in A1 and C1 apply to A1–7 and C1–7, respectively (representative of total of 75 cells for each treatment group, n = 3).
were pretreated for 30 minutes with proteasome inhibitors MG132 (5 μmol/L), Lactacystin (10 μmol/L), or Epoxomicin (25 nmol/L) and cultured with CDDP for an additional 12 hours. Although the proteasome inhibitors displayed no effect on basal FLIP content, they significantly attenuated the decrease in FLIPc and FLIPs level induced by CDDP (Fig. 1B, bottom), suggesting that proteasomal degradation may be responsible for the decreased FLIP content after CDDP challenge. Similarly, CDDP induces the proteasomal degradation of the antiapoptotic protein Xiap without affecting its mRNA abundance (15).

CDDP enhances FLIP-Itch interaction and FLIP ubiquitination. Because proteasomal degradation of most proteins is preceded by ubiquitination, we examined whether CDDP induces FLIPc and FLIPS ubiquitination. OV2008 and A2780s cells were transfected with HA-ubiquitin (2 μg; 24 hours); infected (MOI=25; 24 hours) with adenoviral V5-FLIPc, V5-FLIPS, or LacZ (as control); and treated with CDDP (0–10 μmol/L) in the presence of Epoxomicin (25 nmol/L) for 1.5 and 3 hours, respectively. Ubiquitinated FLIP was immunoprecipitated with anti–HA-ubiquitin and immunoblotted with anti–V5-Fc or anti–V5-Fc. Although FLIP ubiquitination was not detectable with nonspecific IgG or in cells infected with LacZ or HA-ub; alone, CDDP enhanced FLIPc and FLIPS ubiquitination (Fig. 1C–D, bottom), demonstrating that CDDP-induced FLIP degradation in ovarian cancer cells is associated with FLIP ubiquitination.

Because Itch possesses E3 ubiquitin ligase activity and is involved in protein ubiquitination (5), we determined whether Itch plays a role in CDDP-induced FLIP ubiquitination by targeting Itch with siRNA (Fig. 1C–D, top). Although CDDP increased FLIPc and FLIPS ubiquitination in cells transfected with control siRNA, these responses were markedly suppressed by Itch depletion (Fig. 1C–D, bottom), indicating a requirement for Itch in CDDP-induced FLIP ubiquitination. Although Itch has been shown to facilitate FLIPc degradation in hepatocytes (5), FLIPS ubiquitination was not examined nor was the effect of CDDP on FLIPS ubiquitination investigated.

Because the present study is the first to show that Itch plays a vital role in the ubiquitination of both FLIPc and FLIPS after CDDP treatment.

To determine whether Itch directly interacts with FLIP and if such an interaction leads to CDDP-dependent FLIP decrease, coprecipitation studies were carried out with OV2008 and A2780s cells transfected with HA-ubiquitin (2 μg; 24 hours) and subsequently infected (MOI=25; 24 hours) with adenoviral V5-FLIPc, V5-FLIPS, or LacZ and treated with CDDP (0–10 μmol/L) in the presence of Epoxomicin (25 nmol/L). As shown in Fig. 2A and C, FLIPc-Itch interaction was not detected with nonspecific IgG or in cells infected with LacZ or HA-ub alone, CDDP enhanced FLIPc and FLIPS ubiquitination (Fig. 1C–D, bottom), demonstrating that CDDP-induced FLIP degradation in ovarian cancer cells is associated with FLIP ubiquitination.

We further elucidated whether CDDP-induced FLIP ubiquitination is p53 dependent by depleting p53. Our coprecipitation studies show that CDDP enhances p53-FLIP interaction (Fig. 3B–C). These responses were associated with increased CDDP-induced FLIPc and FLIPS ubiquitination but attenuated after p53 down-regulation by p53 siRNA (100 nmol/L; 24 hours; Fig. 3B–C). Interestingly, p53 has been shown to facilitate FLIPc degradation and ubiquitination in colon cancer cells (19) and temperature-sensitive Germ cell-2 (20). These studies are consistent with our contention that, in response to CDDP, p53 binds to FLIP and Itch to facilitate FLIP ubiquitination and proteasomal degradation.

Our results clearly show that p53 plays an important role in CDDP-induced FLIP ubiquitination and proteasomal degradation. If the above hypothesis, we examined FLIP-p53-Itch colocalization by triple immunolabeling on OV2008 cells treated with or without CDDP (0–2.5 μmol/L; 2 hours; Fig. 4). In control cells (DMSO; Fig. 4), FLIP, Itch, and p53-immunoreactivities (-IR) mostly displayed diffuse staining throughout the cytoplasm and at the cell membrane. Individual clusters of FLIP, Itch, and p53 along with a few triple colocalizations were observed in the cytoplasm. In contrast, cells treated with CDDP exhibited numerous FLIP, Itch, and p53 clusters at the cell membrane, with frequent triple colocalizations. Interestingly, CDDP induces p53 nucleus localization at 6 hours but not earlier (data not shown). Our findings are consistent with the contention that FLIP is recruited by Itch at the cell membrane in a p53-dependent manner.

A similar recruitment phenomenon for FLIP by FADD at the Fas receptor has been reported and is believed to suppress caspase-8 activation (2). These results strongly argue in favor of a mechanism involving FLIP-p53-Itch colocalization after CDDP treatment. Moreover, p53, as a docking protein, could facilitate FLIP-Itch interaction, FLIP ubiquitination and degradation, and ultimately apoptosis.
In summary, we report that (a) CDDP down-regulates FLIP protein content via FLIP proteasomal degradation, (b) CDDP enhances FLIP-p53-Itch interaction and FLIP ubiquitination, and (c) p53 and Itch are required for CDDP-induced FLIP ubiquitination. These results are of high relevance for the modulation of FLIP as a possible modality in overcoming chemoresistance in ovarian cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
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