Up-regulation of FLIP in cisplatin-selected HeLa cells causes cross-resistance to CD95/Fas death signalling

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Cisplatin-selected cervix carcinoma HeLa cell lines induced less apoptosis, and weaker activation by cisplatin or Fas-activating antibody, of mitochondrial-associated caspase-9 and death receptor-mediated caspase-8 than did parental cells. Furthermore, less DISC (death-inducing signalling complex) was formed in cisplatin-selected cell lines than in parental cells. Ac-IETD-CHO (acetyl-Ile-Glu-Thr-Asp-aldehyde), which has a certain preference for inhibiting caspase-8, or Fas-antagonistic antibody, significantly inhibited cisplatin-induced apoptosis in both parental and cisplatin-selected HeLa cell lines. These results imply that cell-surface death signalling is inducible by cisplatin; that reduction of this pathway is associated with drug resistance, and that cisplatin-selected cells acquire cross-resistance to cell-surface death signalling. Sequential up-regulation of FLIP (FLICE-like inhibitory protein), but not Bcl-2, Bcl-x_L or inhibitors of apoptosis protein (IAPs), was observed in resistant cells but not in parental cells. The inhibition of FLIP by FLIP antisense oligonucleotides promotes cisplatin and Fas-antibody-induced apoptosis. However, the modulation of apoptosis by FLIP antisense oligonucleotides in resistant cells is greater than that in parental cells. The presented data reveal that the up-regulation of FLIP may contribute to the suppression of apoptosis and thereby change cells that are resistant to cisplatin and Fas-mediated death signals. The results also show that cancer cells that have undergone long-term chemotherapy and become chemoresistant may change the FLIP level, becoming cross-resistant to death factors such as Fas.

Key words: apoptosis, cisplatin, cross-resistance, Fas, FLIP (FLICE-like inhibitory protein).

INTRODUCTION

Although substantial biochemical changes have been observed in cell lines that have become resistant to cisplatin [*cis*-diammine-dichloroplatinum (II)] [1–3], the involvement of apoptotic signalling in cellular resistance to this drug remains unknown. Two of the best-characterized biochemical events during apoptosis are the activation of caspase-3 protease and the fragmentation of DNA into nucleosomal fragments. The literature implies that mitochondria may be crucial in apoptosis because they release cytochrome *c* [4,5]. Cytochrome *c* is associated with a complex of Apaf-1 (apoptotic protease-activating factor 1) and caspase-9, and thus activates caspase-3 [6–8]. Nucleosomal DNA fragmentation occurs during apoptosis, after the activation of the DFF (DNA fragmentation factor), a heterodimeric protein that functions downstream of caspase-3. DFF includes a DFF (40 kDa DFF) and its inhibitor DFF45, which is removed after caspase is cleaved [9]. However, the multimerization of Fas on the cell surface may cause the recruitment of the signalling molecules, FADD (Fas-associated death domain) and caspase-8 to the activated receptor, forming the DISC (death-inducing signalling complex) [10–14]. The oligomerization of caspase-8 within the DISC results in its autoactivation by proteolysis [15], before proteolytic cleavage mediates the activation of other caspases, including caspase-3 [16,17]. These downstream caspases cleave the death substrates, including DFF [18–20]. Caspase-3 is known to be a downstream effector of caspases 8 and 9 in the apoptosis pathway [21–23]. However, in some cases, caspase-3 cleaves and activates caspases 8 and 9 using a feedback mechanism [24]. Mitochondria-mediated apoptotic signalling downstream of the DISC-associated activation of caspase-8 has been considered to be involved in the triggering of apoptosis by cisplatin [21,25], or by Fas or TNFR1 [TNF (tumour necrosis factor) receptor 1] [26,27]. However, the precise role of cell-surface-mediated death signalling in determining cell sensitivity and resistance to cisplatin has not been established.

With respect to intracellular resistance mechanisms, FLIP (FLICE-like inhibitory protein) has been identified as a blocker of apoptosis induced by death receptors of the TNF family [28]. During the early steps of TNF signalling [28], FLIP binds to and neutralizes adaptor proteins and procaspases, which are usually recruited to the cytosolic domains of apoptosis-inducing TNF receptors upon stimulation by ligands. Furthermore, the overexpression of FLIP in cancers has been reported [29]. FLIP prevents the recruitment and cleavage of caspase-8 at the DISC and subsequently inhibits apoptosis in cell types I and II [30].

The cell-surface-mediated Fas signalling pathway can be activated by UV radiation in various types of cell [31]. The authors recently found that mitochondrion-mediated apoptosis induced by cisplatin treatment is reduced in cisplatin-selected HeLa cells [32], which exhibit more DNA repair and recognition of damaged DNA, than in parental cells; cisplatin-selected HeLa cells are also cross-resistant to UV [33,34]. We hypothesize here that Fas signalling may be altered in these resistant cells. The results of this work indicate that cisplatin-selected HeLa cells also acquired cross-resistance to Fas receptor-mediated apoptosis. Moreover, the up-regulation of FLIP in resistant cells may contribute to the suppression of apoptosis, making cells resistant to cisplatin and Fas-mediated death signals.

Abbreviations used: FLIP, FLICE-like inhibitory protein; TNF, tumour necrosis factor; TNFR1, TNF receptor 1; DISC, death-inducing signalling complex; DAPI, 4′,6-diamidino-2-phenylindole; IAP, inhibitor of apoptosis protein; DFF, DNA fragmentation factor; ASO, antisense oligonucleotide; FADD, Fas-associated death domain; Ac-IETD-CHO, acetyl-Ile-Glu-Thr-Asp-aldehyde; Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde.

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MATERIALS AND METHODS

Cell lines and culture

Human cervix carcinoma HeLa cells and cisplatin-selected HeLa cell lines with increasing cisplatin resistance (HeLa-CPR), including R1, R2 and R3 [3], were maintained in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD, U.S.A.) supplemented with 10 % fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco). Cisplatin-resistant clones were generated by stepwise addition of cisplatin and maintained for several weeks, and clones were selected by the factors such as increased DNA repair and GSH, and reduced drug uptake and adducts formation [3,35]. The cell lines were maintained at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air. Cisplatin (1 μM; Farmitalia, Milano, Italy) was added to the medium to maintain the resistant phenotype. Before any experiments, resistant cells intended as source materials were cultivated in media without cisplatin for 3 weeks.

Western blot analysis

Cells (2×10^6) were treated with cisplatin or Fas antibody (clone CH11; Upstate Biotechnology, Lake Placid, NY, U.S.A.), washed twice with PBS and lysed in RIPA lysis buffer [50 mM Tris/HCl, pH 7.4, 1 % Nonidet P-40, 0.25 % sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, protease inhibitor cocktail (Roche, Mannheim, Germany), 1 mM Na_3VO_4 and 1 mM NaF] on ice for 30 min. Insoluble material was removed by centrifugation at 16 000 ***g*** for 10 min at 4 °C. Protein concentrations were measured using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, U.S.A.). Proteins were separated by SDS/PAGE (12 % gels), transferred on to PVDF membranes and incubated with antibodies reactive to caspase-9, caspase-8 (Cell Signalling Tech, Beverly, MA, U.S.A.), caspase-3, DFF, FLIP, Fas, TNFR1 or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). The antigen–antibody complexes were visualized by standard enhanced chemiluminescence reaction (Pierce, Rockford, IL, U.S.A.).

Analysis of DISC

DISC analysis was performed using a standard method [36]. Briefly, after stimulation, cells were washed twice in ice-cold PBS, and lysed in lysis buffer [30 mM Tris, pH 7.5, 150 mM NaCl, 1 mM PMSF, protease inhibitor cocktail, 1 % Triton X-100 and 10 % (v/v) glycerol] for 30 min on ice. After centrifugation at 16 000 ***g*** at 4 °C, an anti-human Fas antibody (1 μg) and Protein A–Sepharose (Amersham Biosciences, Uppsala, Sweden) were added and reacted at 4 °C overnight. The protein–antibody–beads complex was washed three times in lysis buffer, and proteins were eluted from the beads by incubation in boiling water for 5 min in the presence of 5 % 2-mercaptoethanol. After centrifugation, supernatants were used for determination of DISC molecules.

Analysis of viability and apoptosis

For clonogenic survival, cells were seeded in 60 mm dishes and treated with various concentrations of Fas antibody or TNF-α (BD PharMingen, San Diego, CA, U.S.A.). After a 14 day incubation at 37 °C, plates were stained with a Crystal Violet, and colonies with more than 50 cells were scored. For assessment of apoptosis, cells growing in six-well plates were either left untreated or treated with cisplatin, Fas antibody or TNF-α for 24 h. In some cases cells were pre-treated with Ac-IETD-CHO (acetyl-Ile-Glu-Thr-Asp-aldehyde), Ac-DEVD-CHO (acetyl-Asp-Glu-Val-Asp-aldehyde; Bachem, Bubendorf, Switzerland), or Fas-antagonistic antibody (clone G254-274; BD PharMingen) for 1 h and continuously treated together with the indicated concentrations of drug. The cells were fixed with methanol and incubated with DAPI (4′,6-diamidino-2-phenylindole; Sigma) solution for 30 min in darkness. Floating cells from each well were also fixed and returned to the respective wells. All cells were analysed using an Olympus microscope at 420 nm. Apoptotic cells exhibiting morphological features of apoptosis, including chromatin condensation and nuclear fragmentation [37], were counted in six to eight randomly selected fields. Approx. 500 nuclei were examined for each sample, and the results were expressed as the number of apoptotic nuclei over the total number of nuclei counted.

Transfection of cells with ASOs (antisense oligonucleotides)

For antisense experiments, phosphothioated FLIP ASO (5′-ACTTGTCCCTGCTCCTTGAA-3′) or control phosphothioated oligonucleotide (5′-GGATGGTCCCCCCTCCACCAGGAGA-3′; synthesized by the PAN Facility, Stanford University, Stanford, CA, U.S.A.) was delivered in to cells by lipofection (Invitrogen) at a final concentration of 600 nM, as described in [38]. After 4 h, medium was removed and replaced with the appropriate cell growth medium containing the indicated concentrations of oligonucleotide. Cells were incubated for an additional 10 h at 37 °C and then stimulated with the appropriate concentration of cisplatin or Fas antibody for 24 h.

RESULTS

Reduced cell-surface-mediated caspase activation and apoptosis in cisplatin-resistant cells

Cisplatin induced the activation of caspase-9 in a dose-dependent manner in HeLa cells, but this activation was lower in resistant cells (Figure 1A). Cisplatin also induced the activation of caspase-8 in HeLa cells; however, the activation was less in cisplatin-selected cells (R1, R2 and R3) than in parental cells. Caspase-8 was present mainly as two isoforms of approx. 54 kDa, possibly caspase-8a and -8b [39]. Caspase-3 was cleaved proteolytically using cisplatin (40 μM) in HeLa cells, from its inactive precursor form into active fragments, but this treatment either completely failed to activate or only slightly activated caspase-3 in the resistant cells (R2 and R3), to an extent governed by their resistance.

Chromosomal fragmentation involves the activation of DFF and so was also studied. The intensity of the DFF precursor was reduced by cisplatin in HeLa, implying stronger DFF activation; in addition, DFF was activated only slightly in R1 cells, but not in R2 or R3 cells. Chromatin condensation and nuclear fragmentation also demonstrated cisplatin-induced apoptosis. As shown in Figure 1(B), the percentage of HeLa cells that were apoptotic increased dose-dependently; for example 10 and 40 μM cisplatin induced 11 and 51 % apoptosis, respectively. However, the induction of apoptosis was significantly reduced in cisplatin-resistant cells: 40 μM cisplatin induced 28, 17 and 12 % apoptosis in R1, R2 and R3, respectively. Kinetic studies revealed that caspase-8 was activated after 12 h and the activation was strengthened time-dependently in 40 μM cisplatin-treated parental HeLa cells, but the activation was either strengthened only slightly or was weakened greatly in cisplatin-resistant cells (Figure 1C). Consistent with caspase-3 activation at 12 h, DFF cleavage occurred after 12 h in parental HeLa cells, but was not clearly detected in resistant cells. These results reveal that

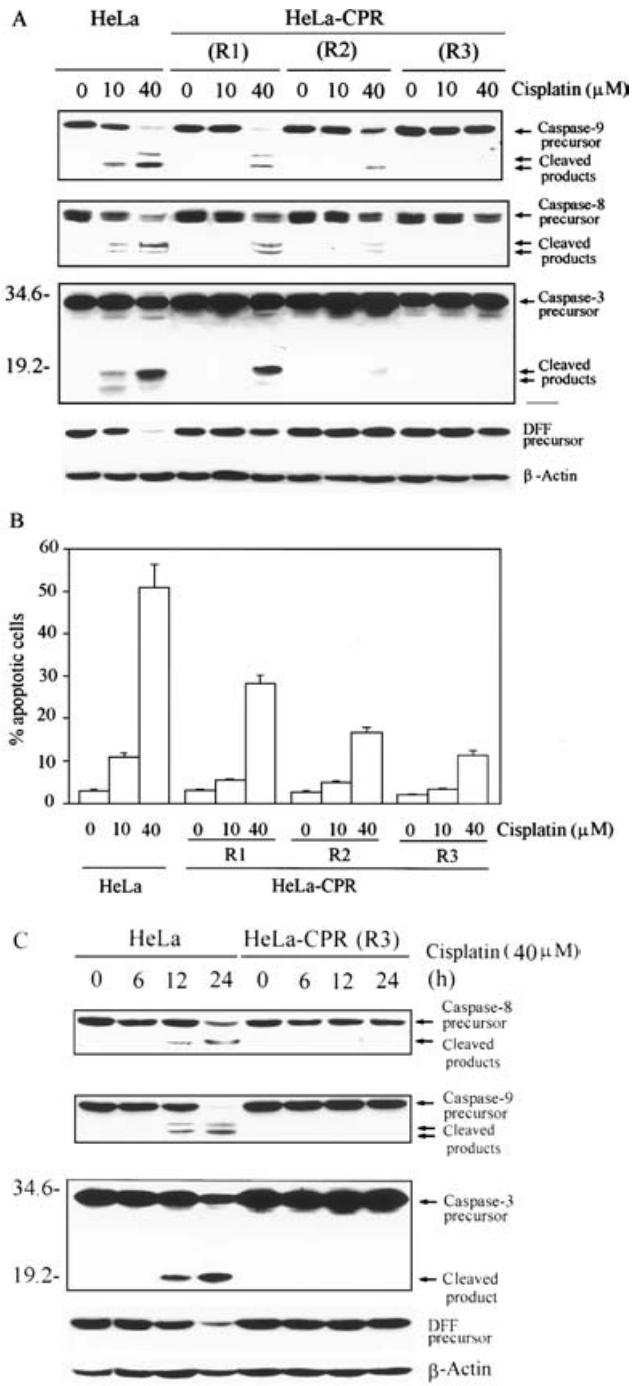

Figure 1 Reduced induction of apoptosis and activation of caspases in cisplatin-resistant cells

HeLa and cisplatin-resistant HeLa cells (HeLa-CPR), R1, R2 and R3, were either left untreated or treated with cisplatin as indicated for 24 h. (**A**) Immunoblot analysis of caspase-9, -8, -3 or DFF. To demonstrate equal protein loading, blots were reprobed with anti-β-actin. The molecular-mass markers (in kDa) are shown on the left. (**B**) Apoptosis was evaluated by DAPI staining and fluorescence microscopy. The plotted values represent the mean $\pm$ S.D. obtained in three independent experiments. (**C**) Kinetic analysis of immunoblotting of caspase-9, -8, -3 or DFF. Cells were treated with cisplatin (40 μM), harvested at the indicated times and immunoblotted using specific antibodies.

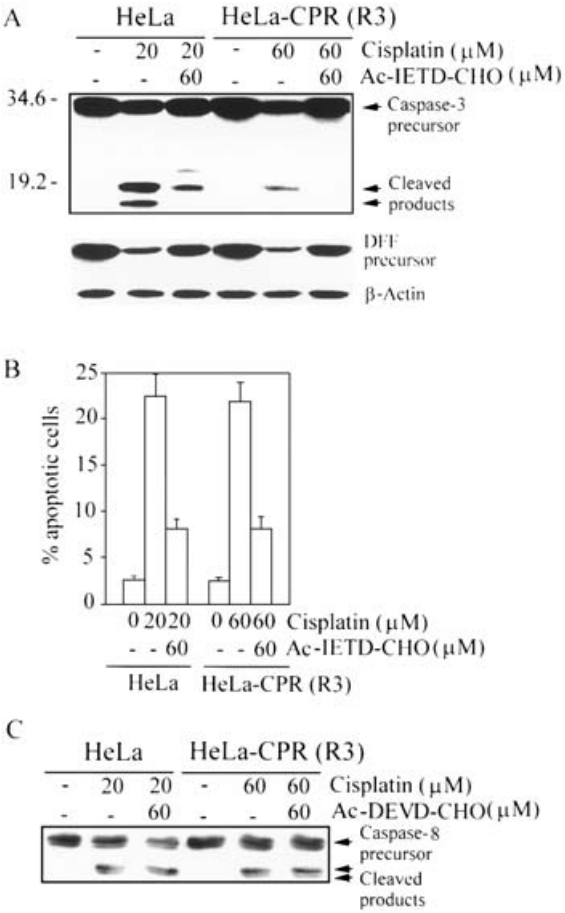

Figure 2 Inhibition of cisplatin-induced caspase-3 activation and apoptosis by caspase-8 inhibitor

(**A**) Inhibition of cisplatin-induced activation of caspase-8 reduced the activation of caspase-3 in both cells. HeLa and HeLa-CPR (R3) cells were either left untreated or pretreated with Ac-IETD-CHO for 1 h, and were continuously treated together with indicated concentrations of cisplatin for 24 h. Immunoblot analysis of the lysates was performed using anti-caspase-3 or anti-DFF. To demonstrate equal protein loading, blots were reprobed with anti-β-actin. The molecular-mass markers (in kDa) are shown on the left. (**B**) Inhibition of cisplatin-induced caspase-8 activation suppressed apoptosis in both cells. Cells were treated as in (**A**), and apoptosis was evaluated by DAPI staining and fluorescence microscopy. The plotted values represent the mean $\pm$ S.D. obtained in three independent experiments. (**C**) Inhibition of caspase-3 activation did not impact the activation of caspase-8. Cells were either left untreated or pretreated with Ac-DEVD-CHO for 1 h and continuously treated together with indicated concentrations of cisplatin for 24 h. Immunoblot analysis of the lysates was performed using anti-caspase-8.

cisplatin may have caused the activation of caspase-8 and perturbation of the mitochondria, leading to activation of the apoptosome. Cisplatin-resistant cells exhibited reduced activation of mitochondria- and cell-surface-mediated caspases. Ac-IETD-CHO, which has a certain preference for inhibiting caspase-8, was included in the typical caspase activation assay to verify these findings. As presented in Figures 2(A) and 2(B), caspase-3 was strongly activated in HeLa and HeLa-CPR (R3) cells treated with 20 and 60 μM cisplatin, respectively. Ac-IETD-CHO markedly inhibited the cisplatin-induced activation of caspase-3, DFF and apoptosis in both cell lines. Interestingly, apoptosis was inhibited by over 50% in both cell lines. Ac-DEVD-CHO, which has a certain preference for inhibiting caspase-3, did not significantly influence the cisplatin-induced caspase-8 activation in either cell line (Figure 2C). These results indicate that the activation of caspase-8 participates in cisplatin-induced apoptosis, and that cell-surface death signalling influences cisplatin resistance upstream of caspase-3.

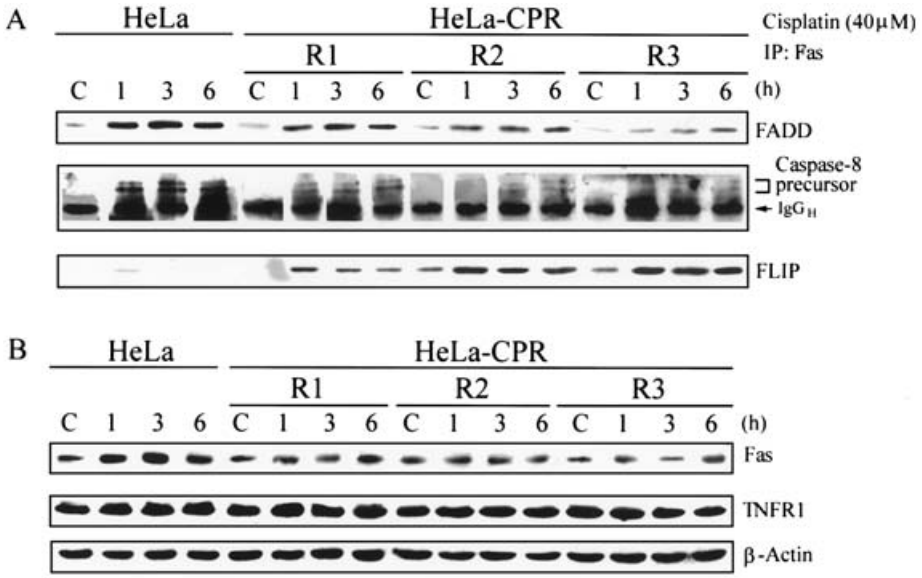

Figure 3 Suppressed induction of DISC in cisplatin-resistant cells

Cells were treated with cisplatin (40 μM), harvested at the indicated times and lysates were immunoprecipitated with Fas antibody. (**A**) Immunoblot analysis of the immune complex performed using anti-FADD (top panel), anti-caspase-8 (middle panel) or anti-FLIP (bottom panel). (**B**) Kinetic analysis of immunoblotting of Fas or TNFR1. Cells were treated with cisplatin (40 μM), harvested at indicated times and immunoblotted using specific antibodies. To demonstrate equal protein loading, blots were reprobed with anti-β-actin. C, control with no cisplatin treatment.

Suppressed induction of DISC in cisplatin-resistant cells

Fas-associated FADD, caspase-8 and FLIP were assayed by co-immunoprecipitation following cisplatin treatment, to determine whether cisplatin-resistant cells affect the formation of DISC. Interestingly, the results of DISC formation assays showed that more FADD and caspase-8 were co-immunoprecipitated with Fas in parental cells than in resistant cells, and that the DISC formation declined as resistance to cisplatin increased (Figure 3A). In contrast, Fas-associated FLIP, which interferes with the activation of caspase-8 at the DISC, increased with resistance of cisplatin. Furthermore, the expression of death receptors, Fas and TNFR1, was detected using specific antibodies. Cisplatin (40 μM) induced marked up-regulation of Fas in HeLa cells, peaking at 3 h (Figure 3B). However, the up-regulation of Fas in resistant cells was less than that in parental cells. The level of TNFR1 was not changed by cisplatin treatment. Cisplatin-resistant cells thus induced less DISC and Fas.

Reduced Fas-mediated activation of caspases and apoptosis in cisplatin-resistant cells

Reduced induction of DISC and activation of caspase-8 by cisplatin in resistant cells raises the possibility that acquired cisplatin resistance may affect membrane receptor pathways. A Fas-mediated apoptotic-signalling pathway in HeLa and HeLa-CPR cells (R1, R2 and R3) was investigated to test this hypothesis. Activating antibodies to Fas activated caspases 8, 9 and 3 and DFF dose-dependently, in both sensitive and resistant HeLa cells (Figure 4A). Interestingly, however, the activation of these proteins was lower in resistant cells (R1, R2 and R3). Fas antibody also up-regulated the expression of Fas in both sensitive and resistant cells. However, the up-regulation was weak in resistant cells (Figure 4B). Fas-induced apoptosis was evaluated with reference to chromatin condensation and nuclear fragmentation: the percentage of apoptotic cells within each cell type increased with dose, but declined from 65 to 25% in HeLa, R1, R2 and R3 cells treated with 30 ng of Fas antibody (Figure 4C). Similarly, TNF-α-induced apoptosis was also lower in cisplatin-resistant cells (results not shown).

The involvement of a Fas pathway in apoptosis was also tested using Fas-antagonistic antibody (2 μg/ml) that effectively reduced Fas-induced apoptosis (Figure 5). Furthermore, colony-forming assay indicated that cisplatin-resistant cells are resistant to Fas-antibody and TNF-α-induced cytotoxicity (Figure 4D and results not shown). These results show that the cisplatin-resistant cells become cross-resistant to death factors.

Inhibiting cisplatin-induced apoptosis by Fas-antagonistic antibody

HeLa cells and cisplatin-resistant HeLa cells (R3) were pre-treated with Fas-antagonistic antibody and continuously treated together with cisplatin to verify the involvement of Fas-mediated signalling in cisplatin-induced apoptosis. The presence of Fas-antagonistic antibody in the medium reduced the equitoxic concentration of cisplatin-induced apoptosis from 30 to 15% in both HeLa and HeLa-CPR (R3) cells (Figure 5). The presence of additional Ac-DEVD-CHO further weakened cisplatin-induced apoptosis equally in both cell lines. For comparison, Fas-antagonistic antibody also reduced Fas antibody-induced apoptosis from 45 to 16% in HeLa cells. The results imply that a Fas-dependent pathway may also mediate cisplatin-induced apoptosis, and Fas-pathway inhibitors in the resistant cells typically block cisplatin-induced apoptosis.

Overexpression of FLIP in resistant cells and sensitization of cisplatin-induced apoptosis by inhibition of FLIP

The expression of anti-apoptotic proteins such as Bcl-2, Bcl-x_L, IAPs (inhibitor of apoptosis proteins) and FLIP in both parental and resistant HeLa cells was examined to elucidate the involvement of the negative regulators of apoptosis in cisplatin-resistance. Interestingly, the expression of FLIP was found to be higher in

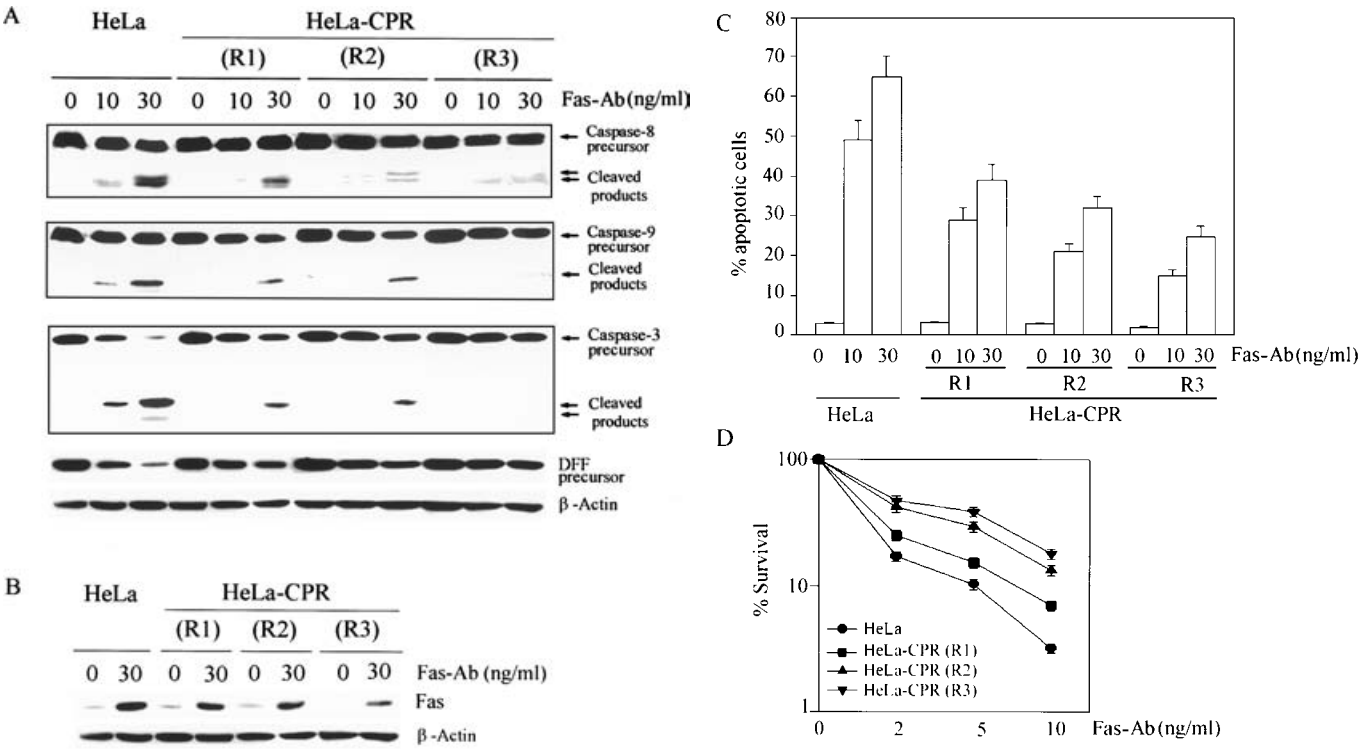

Figure 4 Suppressed Fas-mediated activation of caspases and apoptosis in cisplatin-resistant cells

HeLa and cisplatin-resistant HeLa (HeLa-CPR; R1, R2 and R3) cells were either left untreated or treated with indicated concentrations of Fas antibody [plus cycloheximide (1 μg/ml)] for 24 h. (**A**) Immunoblot analysis of caspase-8, -9, -3, DFF or (**B**) Fas. To demonstrate equal protein loading, blots were reprobed with anti-β-actin. (**C**) Apoptosis was evaluated by DAPI staining and fluorescence microscopy. The plotted values represent the means $\pm$ S.D. obtained in three independent experiments. (**D**) Reduced Fas-antibody-induced cytotoxicity in cisplatin-resistant cells. HeLa and cisplatin-resistant HeLa (HeLa-CPR; R1, R2 and R3) cells were either left untreated or treated with indicated concentrations of Fas antibody [plus cycloheximide (1 μg/ml)] for 12 h. After 14 days of incubation at 37 °C, plates were stained with Crystal Violet, and colonies with more than 50 cells were scored. Data are expressed as percentage survival relative to the survival of untreated cells, and are means$\pm$S.D. obtained in three independent experiments.

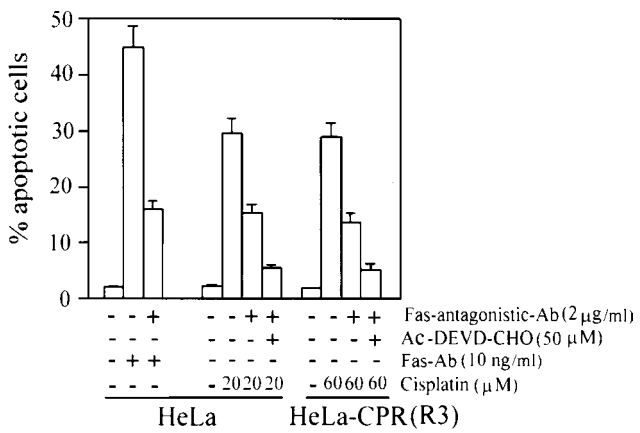

Figure 5 Inhibition of cisplatin-induced apoptosis by Fas-antagonistic antibody

Cells were either left untreated or pretreated with Fas-antagonistic antibody or Ac-DEVD-CHO for 1 h and continuously treated together with indicated concentrations of Fas antibody or cisplatin for 24 h. Apoptosis was evaluated by DAPI staining and fluorescence microscopy. The plotted values represent the means$\pm$S.D. obtained in three independent experiments.

resistant cells than in parental cells (Figure 6A). However, the levels of Bcl-2, Bcl-x_L and IAPs in resistant cells apparently did not differ from those in parental cells (Figure 6A and results not shown). Inhibition of FLIP by FLIP ASO considerably sensitized both parental and resistant HeLa cells to cisplatin-induced apoptosis (Figure 6B), whereas treatment with control oligonucleotide (Figure 6B, CO ASO) did not increase apoptosis induced by cisplatin. Furthermore, FLIP ASO promoted the cisplatin-induced activation of caspase-3 and DFF (Figure 6C). These findings imply that FLIP may protect cells from cisplatin-induced apoptosis and may participate in cisplatin resistance.

Sensitizing Fas antibody-induced apoptosis by inhibiting FLIP

FLIP prevents the recruitment and cleavage of caspase-8 at the DISC and inhibits apoptosis in cells of types I and II [30]. Increased induction of apoptosis and activation of DFF by FLIP inhibiting the response to cisplatin stimuli raises the possibility that FLIP ASO may also modulate the death receptor pathway. As shown in Figure 7, FLIP ASO, but not control ASO, significantly sensitized Fas-mediated apoptosis in both parental and resistant cells (Figure 7A). Consistent with apoptosis, caspase-3 and DFF activation was increased in both parental and resistant cells (Figure 7B).

DISCUSSION

Cisplatin in cultured cells can induce cell-surface death pathways. Notably, the activation of caspase-8 is weaker in serial cisplatin-selected cells than in parental HeLa cells. Other researchers have indicated that caspase activation is a central biochemical event in mitochondrion- and cell-surface-mediated apoptosis [17]. A subfamily of the TNFR superfamily, the death receptors constitute an important system that triggers apoptosis [12–14,40]. Of this

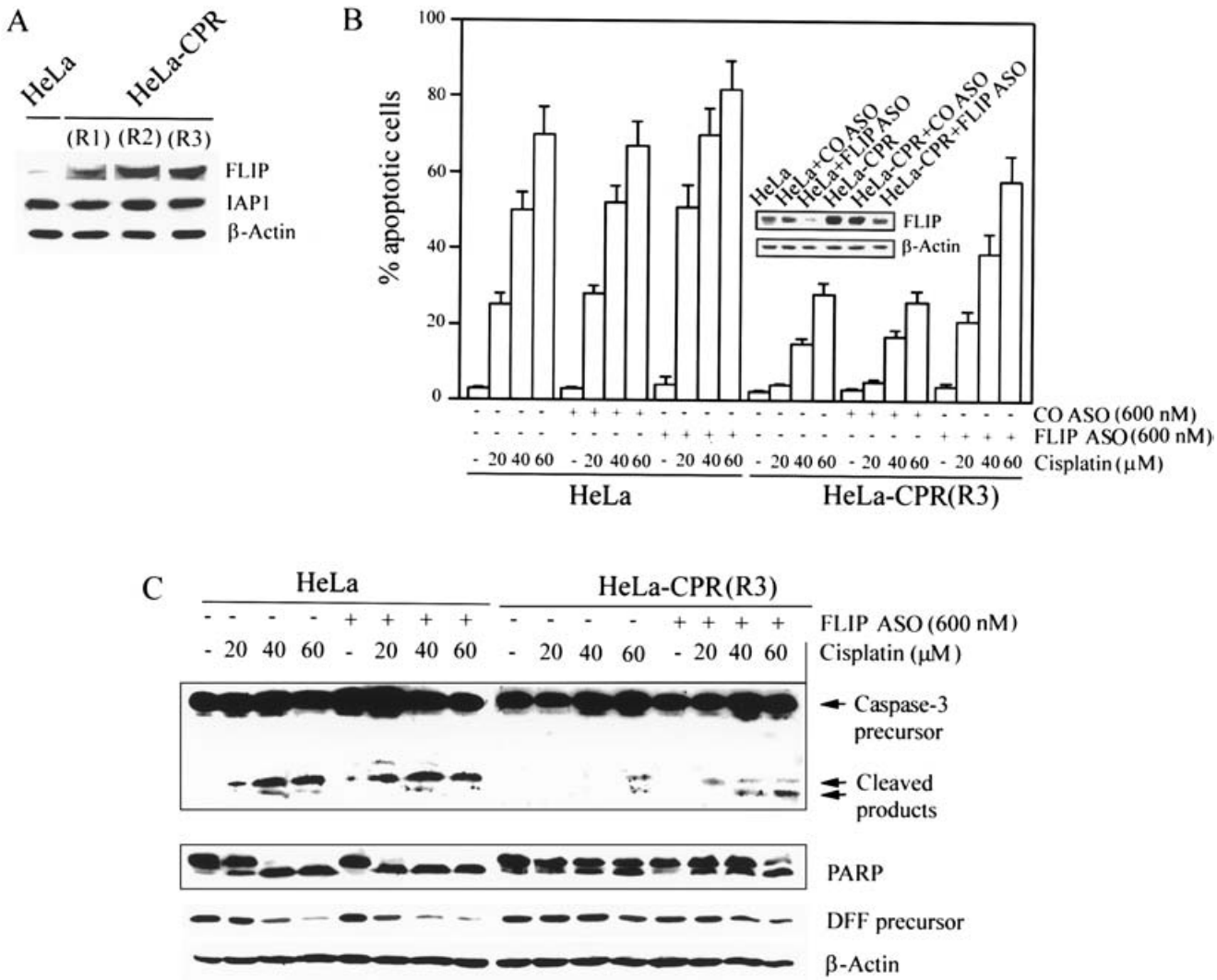

Figure 6 Overexpression of FLIP in cisplatin-resistant cells and sensitization of cisplatin-induced apoptosis by inhibition of FLIP

(**A**) Overexpression of FLIP in cisplatin-resistant cells. Cell extracts of HeLa and cisplatin-resistant HeLa cells (HeLa-CPR), R1, R2 and R3, underwent immunoblot analysis with antibodies to FLIP or IAP1. To demonstrate equal protein loading, blots were reprobed with anti-β-actin. (**B**) Sensitization of cisplatin-induced apoptosis by inhibition of FLIP. HeLa and HeLa-CPR cells were either left untransfected or transfected with control ASO (CO ASO) or FLIP ASO for 14 h, and continuously treated together with indicated concentrations of cisplatin for 24 h. Apoptosis was evaluated by DAPI staining and fluorescence microscopy. The plotted values represent the means $\pm$ S.D. obtained in three independent experiments. The inset indicates the levels of FLIP in cells transfected with control (CO ASO) or FLIP ASOs. (**C**) Increased activation of caspase-3 and DFF by inhibition of FLIP. Lysates were prepared from cells treated as in (**B**) (except CO ASO) and immunoblot analysis was performed using antibodies to caspase-3, PARP (polyADP-ribose polymerase) or DFF.

death receptor family, caspase-8 [41–43], the most receptor-proximal caspase, is recruited to the receptor by the adaptor molecule FADD [44,45]. Treating cells with either cisplatin or Fas-activating antibodies, or with TNF-α, activates caspases 3 and 8 [21,25,27,46,47]. Herein, the activation of caspases 3 and 8 in response to cisplatin is suppressed in cisplatin-selected HeLa cells. Apoptosis in resistant cells is likely to be reduced upstream of caspase-3 because the inhibition of caspase-3 did not influence caspase-8 activation and the inhibition of caspase-8 activity similarly prevents apoptosis in both parental and cisplatin-resistant cells. Evidence that the formation of DISC is suppressed in the resistant cells directly supports this hypothesis. The defect in DISC formation in cisplatin-selected cells by cisplatin confirmed the claim that acquired cisplatin resistance involves a cell-surface-mediated apoptotic pathway. Either the effector caspases in cell-surface-mediated death signals are defective or the regulators that discriminate against the death pathways are strong in resistant cells. Since antagonistic Fas antibody typically attenuates Fas-mediated apoptosis in resistant cells, anti-apoptotic regulators may be acquired therein. Evidence that more Fas-associated FLIP, an inhibitor of caspase-8 activation, was present in resistant cells may support this suggestion. Cisplatin has been recently shown to be able to activate mitochondrion-mediated caspases, but the extent of the activation of caspase is reduced in cisplatin-resistant HeLa cells [32]. Although cisplatin-induced caspase-8 activation in cancer cells has been studied [21,25], this study is the first to demonstrate that the inhibition of a cell-surface-mediated death pathway may be involved in acquiring resistance to cisplatin.

Although the basal level of Fas in resistant cells was similar to that in parental HeLa cells, the induction of Fas and apoptosis was weaker in resistant cells. This result may partially explain the attenuation of the activation of Fas in resistant cells. The lymphoproliferative phenotype was recovered when Fas was expressed as a transgene in the lymphocytes of lpr mice, verifying

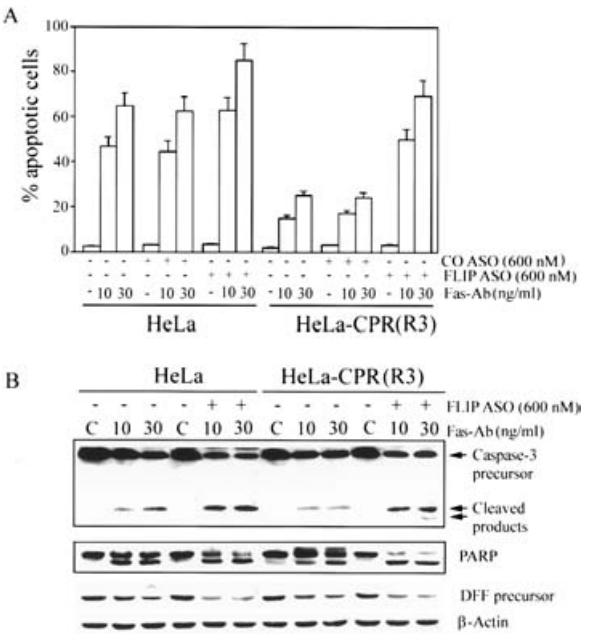

Figure 7 Sensitizing Fas-antibody-induced apoptosis by inhibiting FLIP

(**A**) Sensitizing Fas-induced apoptosis by inhibiting FLIP. HeLa and HeLa-CPR cells were either left untransfected or transfected with control (CO ASO) or FLIP ASO for 14 h and continuously treated together with Fas antibody [plus cycloheximide (1 μg/ml)] for 24 h. Apoptosis was evaluated by DAPI staining and fluorescence microscopy. The plotted values represent the means $\pm$ S.D. obtained in three independent experiments. (**B**) Increased activation of caspase-3 and DFF by inhibition of FLIP. Lysates were prepared from cells treated as in (**A**) (except CO ASO) and immunoblot analysis was performed using antibodies to caspase-3, PARP (polyADP-ribose polymerase) or DFF. To demonstrate equal protein loading, blots were reprobed with anti-β-actin.

that Fas is involved in the apoptosis of T-lymphocytes [48]. UV- or cytotoxic drug-induced apoptosis is much weaker in Fas-resistant cells [31,49,50]. Furthermore, the overexpression of Fas increases the sensitivity of UV-induced apoptosis [31]. Therefore, a chemotherapeutic agent, such as cisplatin, which may activate the Fas pathway, is a potential cause of cross-resistance to other agents that activate Fas. FLIP protein has been identified to block apoptosis induced by death receptors of the TNF family or chemotherapeutic agents [28,51–53]. The suppressed induction of Fas-mediated apoptosis in resistant cells is associated with high levels of FLIP, which interfere with the activation of caspase-8 at the DISC. The modulation by FLIP ASO of cisplatin or Fas antibody-induced apoptosis in resistant cells is stronger than that in parental cells, implying that FLIP up-regulation is an important factor in governing cross-resistance to Fas death signalling. The data imply that the up-regulation of FLIP may make cells resistant to cisplatin. Accordingly, long-term exposure of cells to cisplatin may sometimes induce mutations, such as reduced Fas activation and/or up-regulation of the anti-apoptotic protein, FLIP. Although levels of Bcl-2 and other anti-apoptotic proteins are the same in resistant cells, the possibility of the involvement of active proteins like the phosphorylated form of Bcl-2 [54] cannot be excluded. The failure to undergo apoptosis is frequently linked to drug resistance, so the combined activation of death pathways, including mitochondrion- and cell-surface-mediated pathways, may represent an effective method of killing tumour cells. The evidence presented herein indicates that cancer cells that are exposed for a long time to cisplatin may become resistant to mitochondria and to cell-surface-mediated apoptosis, and that DISC formation and FLIP are potential targets for overcoming drug resistance in cancer treatment.

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