Sensitization of p53-mutated epithelial ovarian cancer to CD95-mediated apoptosis is synergistically induced by cisplatin pretreatment

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Abstract

Epithelial ovarian carcinoma (EOC) remains a highly lethal malignancy. Despite the progress in surgical and therapeutic strategies, resistance to chemotherapy is still a major concern. Cytotoxic therapies mediate killing of cancer cells by activating the intrinsic mitochondrial apoptotic pathway, and p53 status is a key factor in determining the efficacy of apoptotic signaling. The extrinsic (CD95) death receptor–dependent signaling pathway also contributes to the efficacy of cancer therapy. We previously showed that EOC are generally resistant to CD95-dependent apoptosis. In p53 wild-type EOC tumors, CD95-mediated apoptosis is impaired at the receptor level by the long form of cellular FLICE-inhibitory protein, whereas this mechanism does not account for resistance in tumors with mutated p53 (p53mu). In the present study, we examined both intrinsic and death receptor–dependent apoptotic signaling in p53mu OVCAR3 EOC cell line, showing that these cells are less susceptible to cisplatin treatment as compared with p53 wild-type EOC cells and also resist CD95-mediated apoptosis due to inefficient formation of the death-inducing signaling complex and weak mitochondrial signal amplification. However, pretreatment of OVCAR3 cells with clinically relevant cisplatin concentrations significantly improved receptor-dependent apoptotic signaling by up-modulating CD95 receptor expression and increasing death-inducing signaling complex formation efficiency. The synergy of cisplatin pretreatment and CD95 triggering in inducing cell death was also shown in p53mu tumor cells derived from ascitic fluid of advanced-stage EOC patients. These findings support the effectiveness of a combined therapeutic treatment able to sensitize cancer cells to apoptosis even when p53 is functionally inactivated. [Mol Cancer Ther 2007;6(2):762–72]

Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecologic malignancy. The majority of the patients are diagnosed with advanced disease. Although many new reagents have been developed recently, platinum drugs remain the most active in ovarian cancer treatment after surgical debulking. About 20% to 30% of patients are resistant to front-line therapy, and among responders, ~80% will have tumor recurrence and will develop resistance to chemotherapy. Thus, the overall 5-year survival is still <50% (1).

Killing of cancer cells by chemotherapeutic agents or by triggering cell-surface death receptors such as CD95 relies on activation of apoptotic signaling pathways (2, 3). Indeed, the resistance to chemotherapy is due mainly to the failure of tumor cells to undergo apoptosis. Although p53 status plays a leading role in determining the efficacy of apoptotic signaling, tumor cells can evade apoptosis using multiple mechanisms (4, 5). We recently showed that in EOC cells bearing a wild-type (wt) p53, CD95-mediated apoptosis is blocked at the receptor level by recruitment of the long form of cellular FLICE-inhibitory protein (c-FLIP$_L$) to the death-inducing signaling complex (DISC; ref. 6). In these cells, apoptotic signal transduction is restored upon down-modulation of c-FLIP$_L$ expression. Nevertheless, the efficiency of DISC formation in EOC cells is generally low and the mitochondrial pathway is needed to complete CD95 signaling (6, 7). Although activation of caspase-8 at the DISC level is not sufficient to initiate a caspase cascade, it is sufficient to activate the mitochondrial pathway through the Bcl-2 family member Bid. Signal mediated by the truncated form of Bid allows mitochondrial membrane permeabilization, release of cytochrome *c*, apoptosome formation, and eventually activation of execution caspases and cellular substrate cleavage (5). Thus, mitochondria, acting as a signal amplifier for the low caspase activity generated at the DISC (7, 8), represent the critical element in those cells where receptor-mediated apoptosis is unable to activate effector caspases directly.

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Novel therapeutic strategies to overcome resistance to chemotherapeutic treatment are urgently needed, especially in EOC with functionally inactivated p53, which characterizes 40% to 70% of ovarian cancers (9) and renders chemoresistant phenotype (10, 11) more difficult to overcome than in p53wt tumors (12). The ability of various chemotherapeutic drugs to induce or increase CD95 expression in cancer cell lines (13) has stimulated interest in targeting the CD95 molecule to enhance cell death. Although loss of p53 function has been shown to affect CD95 up-regulation in response to chemotherapy (14), there is increasing evidence that TAp63α and TAp73β activate the CD95 promoter instead of p53 (15, 16). In a recent study using EOC cell lines, evidence was obtained that combinations of standard anticancer agents and compounds acting on the apoptotic extrinsic pathway might ultimately increase the response of EOC to drug treatment (17, 18).

In the present study, we show that cisplatin pretreatment increases CD95 expression, DISC formation efficiency, and eventually apoptosis in OVCAR3 ovarian carcinoma cells used as the prototype of p53-mutated (p53mu) EOC. The effectiveness of the combined treatment was further shown in primary ovarian tumor cells derived from ascitic fluid of advanced-stage EOC patients.

Materials and Methods

Cell Lines

Two human serous ovarian carcinoma cell lines were used: OAW42 (kindly provided by Dr. A. Ullrich, Max Planck Institute of Biochemistry, Martinsried, Germany), which carries wt p53 (19), and OVCAR3 (American Type Culture Collection, Manassas, VA), which is p53 mutated.[4] OVCAR3 cells were cultured in RPMI 1640 and OAW42 cells in MEM, both supplemented with 10% FCS and 2 mmol/L glutamine, in a 5% CO_2 humidified atmosphere at 37°C.

Induction of CD95-Mediated Apoptosis

Cells were plated, incubated at 37°C, and left untreated or treated for 24 h with 300 ng/mL of agonistic anti-CD95 antibody (clone CH-11, MBL Co., Ltd., Nagoya, Japan) in the presence or absence of 1 μg/mL cycloheximide (Sigma Chemical Co., St. Louis, MO).

Detection of Apoptosis by Propidium Iodide Staining

Cells treated as indicated were washed twice in cold PBS, resuspended in cold fixation buffer (5 mmol/L glucose, 137 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L Na_2HPO_4, 1 mmol/L K_2HPO_4, 0.7 mmol/L EDTA), and permeabilized with 70% ethanol for 30 min on ice. After two washes with PBS, cells were incubated with 1 mg/mL RNase (Roche, Basel, Switzerland) in PBS for 30 min at 37°C followed by 30-min incubation on ice in PBS containing 20 μg/mL propidium iodide (Sigma). The percentage of cells in different phases of the cell cycle was determined on the FL-3 channel of a FACSCalibur (Becton Dickinson, San Jose, CA) using CELLQuest software. Apoptosis was evaluated as the percentage of cells with a sub-G_0-G_1 DNA content.

Subcellular Fractionation and Cytochrome *c* Release

Cells treated as indicated above were trypsinized and, after two washes with PBS, resuspended in 1 mL of HB-7S buffer [1 mmol/L EGTA Na-free, 5 mmol/L Tris-HCl (pH 7.4), 1 mmol/L DTT, and 11% sucrose]. Cells were passed 10 times through a 26-gauge needle and centrifuged thrice at 1,000 × *g* for 10 min at 4°C. The final pellet corresponds to the nuclear fraction. Supernatants were recovered from each step, pooled, and further centrifuged at 24,000 × *g* for 45 min at 4°C. The pellet obtained was resuspended in HB-7 buffer (HB-7S without sucrose) and centrifuged twice at 24,000 × *g*; the final pellet corresponds to the mitochondrial enriched fraction. Supernatants recovered from each centrifugation step were pooled and further centrifuged at 100,000 × *g* to obtain the cytosolic enriched fraction.

Western Blotting and Immunoprecipitation

Cells treated as indicated were rinsed twice in cold PBS and incubated for 30 min on ice in lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP40, 0.5% sodium desoxicholate, 0.1% SDS] containing a protease inhibitor cocktail (Roche). Detergent lysates were centrifuged (13,000 rpm for 15 min at 4°C), the supernatant was collected, and protein concentration was determined by bicinchoninic acid assay (Pierce, Rockford, IL). Lysates (40 μg) were separated on a 12% or 10% SDS-PAGE gel and blotted on a nitrocellulose membrane (Hybond C-Super, Amersham, Little Chalfont, Buckinghamshire, United Kingdom). Blots were saturated in Blotto (5% nonfat dry milk, 0.1% Tween in PBS) and incubated with the following primary antibodies, diluted in Blotto at the concentration recommended by the manufacturer: rabbit polyclonal antibodies to actin-β (Sigma), CD95 (C-20; Santa Cruz Biotechnology, Santa Cruz, CA), and Bid (Cell Signaling, Danvers, MA); mouse monoclonal antibodies to caspase-8 and caspase-3 (Alexis Biochemicals, Lausen, Switzerland), poly(ADP-ribose) polymerase (PARP; PharMingen, BD Biosciences, San Jose, CA), OxPhos Complex IV subunit IV (Molecular Probes, Carlsbad, CA), cytochrome *c* (from ApoAlert Cell Fractionation Kit, Clontech, Mountain View, CA), tubulin-α (Ab-2, clone DM1A, NeoMarkers, Fremont, CA), and Fas-associated death domain (FADD; PharMingen, BD Biosciences). The relevant secondary antibodies conjugated with horseradish peroxidase (Amersham) and diluted in Blotto were added and reactions were developed by enhanced chemiluminescence (Amersham).

DISC components were immunoprecipitated as previously described (6) in cells unexposed or exposed to subcytotoxic doses of cisplatin. Control cells were incubated with the APO-1 monoclonal antibody (Alexis Biochemicals) after lysis. Immunoprecipitates were separated by 4% to 12% SDS-PAGE and immunoblotted with anti-CD95, anti-FADD, and anti–caspase-8 antibodies.

Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential ($\Delta\Psi_m$) was determined based on retention of JC-1 cationic lipophilic dye

[4] http://p53.free.fr/

(JC-1 Mitochondrial Potential Sensor, Molecular Probes). Briefly, cells treated as indicated were washed twice with PBS and loaded with JC-1 dye (5 μg/mL) in PBS for 10 min at 37°C. Stained cells were analyzed on FACSCalibur (Becton Dickinson) using CELLQuest software. In mitochondria with normal membrane potential, the lipophilic dye JC-1 is retained as aggregates detectable in the red fluorescence channel (FL2), whereas, upon mitochondrial membrane depolarization, JC-1 diffuses into the cytoplasm as monomers detectable only in the green fluorescence channel (FL1). Therefore, mitochondrial membrane depolarization is indicated by a decrease in the red/green fluorescence intensity ratio and is determined as percentage of cells with reduced capacity to retain the dye in the aggregate form.

Caspase-3 Activity Assay

Caspase-3 activity from cytosolic extracts of cells treated as indicated was measured by fluorimetric assays using the synthetic fluorogenic substrate DEVD-AFC (PharMingen). Cleavage specificity was assessed by assays in the presence of the inhibitor DEVD-CHO (PharMingen). After the indicated treatments, cells were washed, lysed, and cytosolic extracts, which were collected by centrifugation, were assayed for caspase activity by incubation for 1 h at 37°C with the fluorogenic substrates. Cytochrome *c*/dATP–dependent activation of caspase-3 was assessed in a cell-free system by adding 10 μmol/L cytochrome *c* plus 1 mmol/L dATP to cytosolic extracts of untreated cells for 30 min at 37°C. Caspase activation was tested by adding DEVD-AFC fluorogenic substrate as described above. Fluorescence was detected using a fluorometer equipped with 400-nm excitation and 505-nm emission filters. Relative AFC fluorescence was normalized to lysate protein concentrations.

Cisplatin Treatments

Cisplatin cytotoxicity was evaluated in cells plated in triplicate at 1×10^4 per well in flat-bottomed 96-well microtiter plates, incubated overnight at 37°C, and incubated for 24 h with different concentrations of cisplatin, ranging from 0.1 to 100 μmol/L (Platinex 25 solution 25 mg/50 mL, Bristol-Myers Squibb, New York, NY), added to each well. The drug was removed and cells were further incubated for 24 h with fresh medium. Cell growth was assessed by sulforhodamine B dye (Sigma) assay. Briefly, cells were fixed with 10% cold trichloroacetic acid added to medium for 1 h at 4°C; after washing in water, fixed cells were loaded with 4% sulforhodamine B in 1% acetic acid for 30 min at room temperature, washed with 1% acetic acid, solubilized in 10 mmol/L Tris (pH 10.5), and analyzed with a spectrophotometer at 550 nm. IC_{30} and IC_{50} were evaluated.

In all other experiments, adherent cells were exposed to 3 μmol/L cisplatin (IC_{30}) for 24 h and cultured in fresh medium for the time indicated. In the case of cisplatin and anti-CD95 cotreatment, cells incubated with cisplatin for 24 h were left untreated or treated for an additional 24 h with 300 ng/mL of agonistic anti-CD95 CH11 (MBL) and processed. The cisplatin and CH11 interaction was analyzed using a method from Drewinko et al. (20), calculating the Drewinko index as SF1 × SF2 / SF1-2, where SF1 and SF2 are the surviving fractions of cells exposed to compounds 1 and 2, respectively, and SF1-2 is the surviving fraction of cells exposed to compound 1 in combination with compound 2. Drewinko index > 1 indicates greater than additive effects (i.e., synergy); Drewinko index = 1 indicates additivity; and Drewinko index < 1 indicates antagonism.

Detection of CD95 Cell-Surface Expression

CD95 expression was evaluated using an anti-CD95-FITC monoclonal antibody (Becton Dickinson) diluted as specified by the manufacturer. Incubation of specific antibody or isotype-matched control was carried out in PBS + 0.03% bovine serum albumin for 30 min on ice. Stained cells were washed in PBS + 0.03% bovine serum albumin and analyzed on FACSCalibur (Becton Dickinson) using CELLQuest software.

Isolation of Triton X-100–Insoluble Fraction

Cells were plated and treated as indicated. After two ice-cold PBS washes, cells were lysed in 1% Triton X-100 lysis buffer as described (21) and centrifuged for 15 min at 13,000 rpm to separate the Triton X-100–soluble (supernatant) and Triton X-100–insoluble (pellet) fractions. Pellets were then lysed in an equal volume of 2% SDS–containing loading buffer. Protein concentration was determined by bicinchoninic acid assay on the Triton X-100 soluble fraction, and a 40-μg corresponding volume of the insoluble fraction was analyzed by Western blotting.

Inhibition of Caspase-9 Activity

Cells were plated and left to adhere for 24 h. Before drug treatment, the cell-permeable, irreversible caspase-9 inhibitor z-LEHD-fmk (Calbiochem, Darmstadt, Germany; ref. 22) was added to the culture medium at a concentration of 50 μmol/L. Cells were grown and treated as described above in the continuous presence of the inhibitor.

Isolation of Tumor Cells from Ascitic Fluid

Ascitic fluids were collected during surgical procedures from EOC patients undergoing debulking surgery at Istituto Nazionale Tumori. Tumor samples were obtained with Institutional Review Board approval from patients at the first diagnosis and not previously treated with chemotherapeutic regimens. All patients gave informed consent to use leftover biological material for investigational purposes. Tumor cells from ascitic fluid were isolated as follows. Briefly, ascitic fluid was centrifuged at 400 × *g* for 10 min at room temperature, then pelleted cells were rinsed in 10-mL culture medium without serum and layered on a discontinuous 75% to 100% Ficoll gradient. After a 30-min centrifugation at 680 × *g* at room temperature, the fraction over the 75% Ficoll was recovered and placed in a T75 flask (Costar, Corning, Corning, NY) for at least 1 h in a 5% CO_2 humidified atmosphere at 37°C to separate non-tumor-adherent cells from tumor cell clumps. Cells growing in suspension (tumor cell clumps) were recovered and treated as described with cisplatin at concentrations ranging from 5 to 10 μmol/L, which were considered clinically relevant (23). Molecular analysis of p53 status was done on genomic DNA extracted from each

sample and screened as described (6). Immunohistochemical analysis of c-FLIP expression has been done essentially as described (6) on tumor samples obtained at the time of ascites collection.

Results

In many tumors, including EOC, resistance to apoptosis is considerably increased by p53 mutation. Indeed, analysis of CD95-mediated apoptosis in cells treated with agonistic anti-CD95 antibody CH11 and the protein synthesis inhibitor cycloheximide revealed a high percentage of cells with sub-G_0-G_1 in the p53wt OAW42 cell population, whereas apoptosis in the p53mu OVCAR3 cells never exceeded background levels (Fig. 1A). Moreover, OVCAR3 cells were 10 times more resistant than OAW42 cells to cisplatin treatment, with IC_{50} of 7.4 ± 0.78 and 0.62 ± 0.04 μmol/L (mean ± SD; n = 6), respectively (Fig. 1B). Consistently, OVCAR3 cells were less prone to drug-dependent apoptosis than OAW42 cells, as shown by the lower percentage of cells that entered the sub-G_0-G_1 phase after cisplatin treatment (Fig. 1C).

CD95 Triggering Cannot Activate the Intrinsic Mitochondrial Pathway in p53mu OVCAR3 Cells

In EOC cell lines, receptor-mediated apoptosis requires mitochondrial amplification to complete death signaling efficiently (6). Activation of the intrinsic pathway is

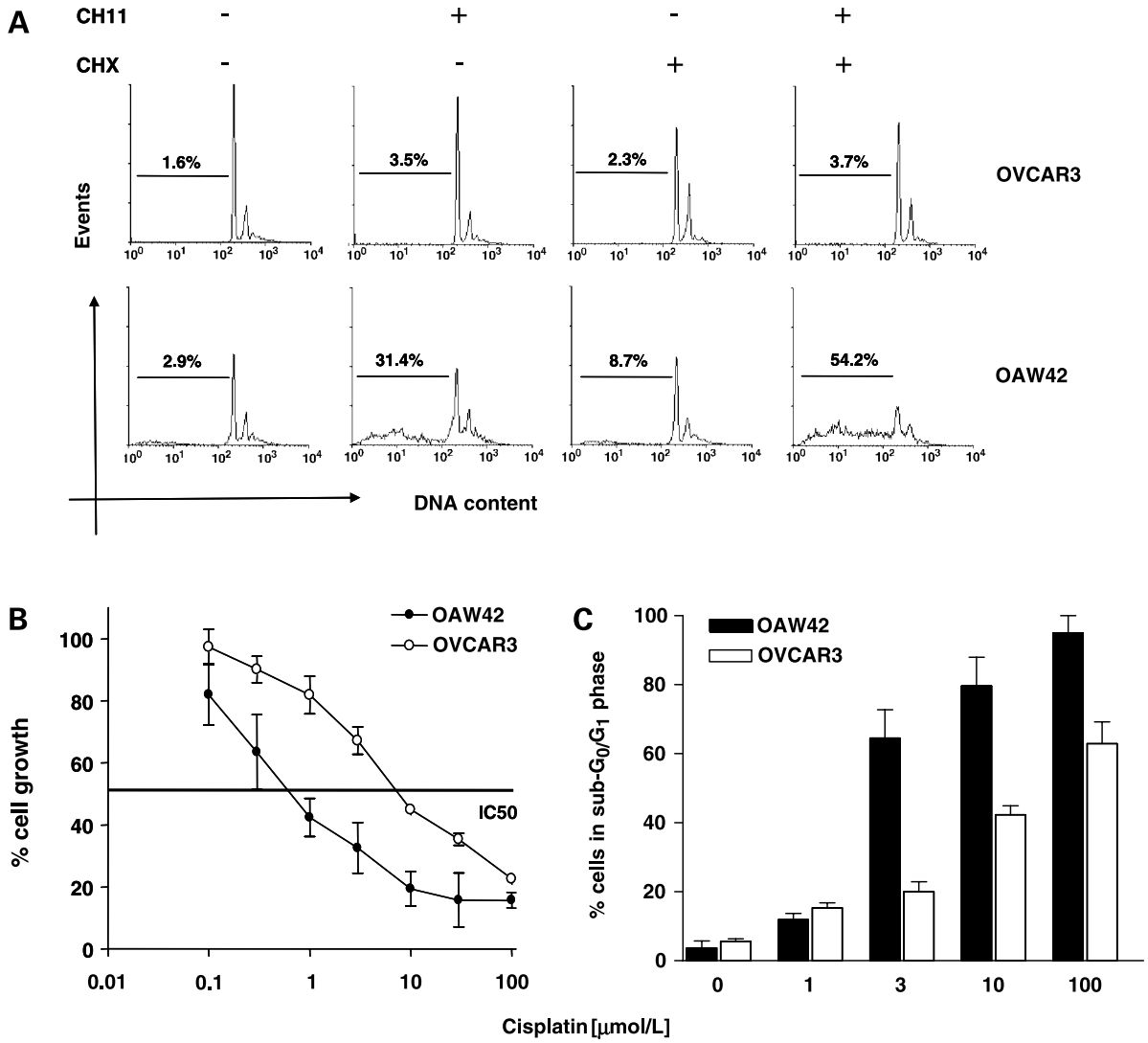

Figure 1. p53mu EOC cells are resistant to CD95-mediated apoptosis and to cisplatin treatment. **A,** OAW42 (p53wt) and OVCAR3 (p53mu) cells were left untreated or treated for 24 h with 300 ng/mL of agonistic anti-CD95 antibody (CH11) in the presence or absence 1 μg/mL of a protein synthesis inhibitor [cycloheximide (*CHX*)]. Percentage of cells with sub-G_0-G_1 DNA content (evaluated by propidium iodide staining) corresponding to apoptotic cells is indicated for each treatment. Results from a representative experiment of three. **B,** cell growth assay done on OVCAR3 and OAW42 cell lines exposed to serial dilutions (100 – 0.1 μmol/L) of cisplatin and evaluated by sulforhodamine B assay. Cell growth percentage was evaluated normalizing to untreated control cells. Results are from six different experiments. **C,** percentage of cells with sub-G_0-G_1 DNA content (evaluated by propidium iodide staining) after exposure of OVCAR3 and OAW42 cell lines to the indicated cisplatin doses. Results are from three different experiments.

detectable by evaluating the decrease in mitochondrial membrane polarization, which can be determined as percentage of cells with reduced capacity to retain the JC1 lipophilic dye in the aggregate form (see Materials and Methods for details). We found that, in the presence of both CH11 and cycloheximide, mitochondrial membrane potential decreased in 44 ± 7.2% of p53wt OAW42 cells whereas membrane depolarization was detected only in 8.3 ± 3.2% of p53mu OVCAR3 cells (mean ± SD; n = 3 experiments; P = 0.0014). A representative experiment is shown in Fig. 2A.

Furthermore, Western blot analysis on subfractionated cell lysates derived from CD95-activated OVCAR3 cells did not reveal cytosolic release of cytochrome *c* (Fig. 2B), suggesting that receptor-dependent apoptotic signaling in these cells is either insufficient to activate the intrinsic pathway or blocked at the mitochondrial level. At variance, in p53wt OAW42 cells, cytosolic release of cytochrome *c* was evident after CD95 triggering (Fig. 2B).

Downstream to mitochondria, cytochrome *c*/dATP–dependent activation of caspase-3 is an indicator of apoptosome functional activity (24). We therefore evaluated caspase-3 activity based on cleavage of the DEVD-AFC substrate using cytosolic extracts of OVCAR3 cells previously incubated with cycloheximide/CH11 and using a cell-free system in which cytochrome *c*/dATP was added to cytosolic extracts from untreated cells. Consistent with the absence of cytochrome *c* release in the cytosol of CD95-triggered OVCAR3 cells, CD95-dependent signaling did not reach the caspase-3 substrate in the cellular system (0.15 ± 0.04 relative fluorescence units/μg of protein). However, a significant DEVD-AFC cleavage (2.9 ± 0.9 relative fluorescence units/μg of protein) was detected in the OVCAR3 cell-free system (Fig. 2C). Such activity is comparable to that observed in the p53wt OAW42 cell-free system (3.4 ± 1.2 relative fluorescence units/μg of protein), suggesting that no further blocks in the apoptotic signal are present downstream to mitochondria in OVCAR3 cells. Data are mean ± SD obtained in three to six different experiments.

Subcytotoxic Doses of Cisplatin Enhance DISC Formation Efficiency in p53mu OVCAR3 Cells by Increasing CD95 Expression Levels and Relocalizing the DISC Components to Lipid Rafts

Exposure of p53mu OVCAR3 cells to subcytotoxic doses of cisplatin (3 μmol/L, IC_{30} corresponding dose), in an attempt to increase CD95-mediated apoptosis, did not induce activation of the intrinsic mitochondrial apoptotic pathway, as indicated by the lack of any significant mitochondrial membrane depolarization. Moreover, no caspase-3 activation was detected and PARP cleavage remained essentially unchanged as compared with untreated cells (see following paragraphs for representative cisplatin activity).

By contrast, cisplatin treatment at subcytotoxic doses induced a time-dependent increase in CD95 expression on OVCAR3 cells, as detected by Western blot analysis on total cell lysates (Fig. 3A). In comparison with untreated cells, we observed an increase of 8.7 ± 4.5% (mean ± SD) in

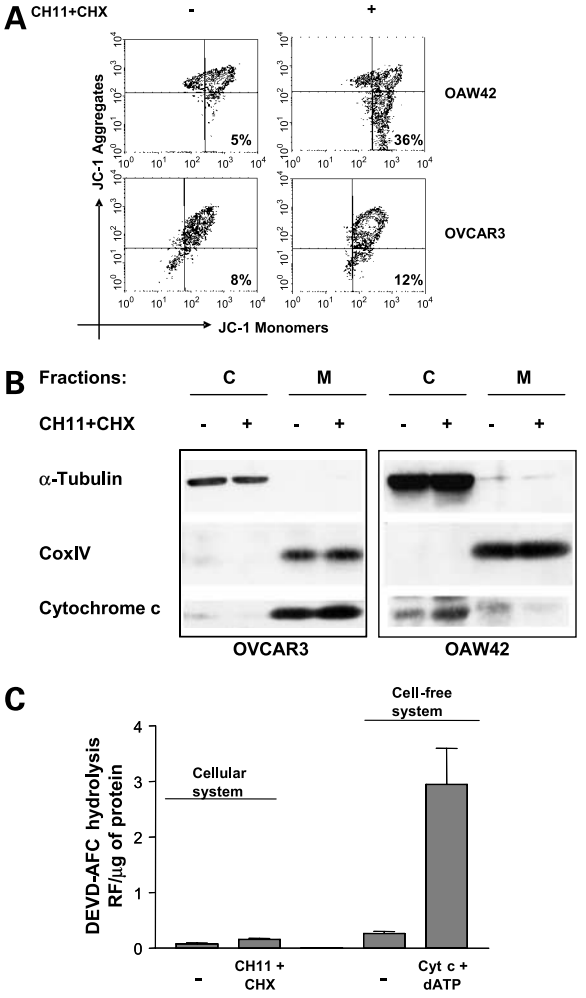

Figure 2. Lack of mitochondrial involvement after CD95 triggering in OVCAR3 cells. **A,** mitochondrial membrane potential as evaluated by JC-1 lipophilic dye incorporation. Mitochondrial membrane depolarization is indicated by a decrease in the red/green fluorescence intensity ratio and is determined as percentage of cells with reduced capacity to retain the dye in the aggregate form, which are detectable in the bottom right quadrant of the dot plot. Representative of three experiments. **B,** Western blot analysis of cytosolic (*C*) and mitochondrial (*M*) enriched fractions (see Materials and Methods) derived from OVCAR3 and OAW42 cells treated with CH11 + cycloheximide or untreated. Proteins were separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane. Blots were probed with anti–cytochrome *c* antibody, with anti–α-tubulin and anti-CoxIV antibodies as purity and loading controls for the cytosolic and mitochondrial fractions, respectively. **C,** caspase-3 activity was determined by cleavage of DEVD-AFC fluorogenic substrate both in a cellular system using cytosolic extracts of treated cells and in a cell-free system using cytosolic extracts in the presence of cytochrome *c* (10 μmol/L) and dATP (1 mmol/L) for 1 h at 37°C. AFC release is expressed as relative fluorescence units (*RF*) per microgram of protein present in the cellular extracts. *Columns,* mean of three to six independent experiments; *bars,* SD.

CD95 expression after 24 h of drug exposure and an average increase of 48 ± 23% when cells were cultured for additional 24 h after drug removal (n = 3 experiments; P = 0.039). It has to be noted that the CD95 expression level observed following cisplatin treatment on OVCAR3 cells

never reached the CD95 constitutive expression observed in OAW42 cells, remaining 35 ± 7.8% lower as determined by densitometric analysis of three different Western blotting experiments. At the cell surface level, the CD95 expression pattern followed the same trend, with fluorescence-activated cell sorting analysis revealing a 1.5- to 2-fold increase in the fluorescence index after 24 h of cisplatin treatment and a 2- to 4.6-fold fluorescence index increase in the following 24 h (Fig. 3B). The CD95 basal expression of the molecule, obtained by keeping the cells in culture with medium alone, remained essentially unchanged at all time points considered.

Immunoprecipitation experiments done on OVCAR3 cells exposed to cisplatin revealed an increased efficiency of DISC formation in treated cells with a more robust recruitment of all components (Fig. 3C). In a representative DISC immunoprecipitation, following receptor triggering, the recruitment of CD95, adaptor molecule FADD, and caspase-8 is increased by 36%, 42%, and 51%, respectively, as compared with untreated cells. As expected, in the control lanes, where the immunoprecipitating agent (APO-1) was added after cellular solubilization, CD95 was the only molecule detectable.

Because the initial phases of CD95 death signaling occur in lipid rafts (25), we examined the recruitment of CD95 and other DISC components into the insoluble plasma membrane region to determine whether cisplatin pretreatment favors a more efficient CD95 relocalization in functional membrane subdomains. Western blotting of the Triton X-100–insoluble fractions (Fig. 3D) of cells exposed to the single-agent treatments showed that cisplatin exposure increased CD95 expression and relocalization (*lane 2*), whereas FADD and caspase-8 were recruited in these regions following receptor triggering (*lane 3*). However, only the combined treatment (cisplatin pretreatment followed by receptor triggering) was able to significantly

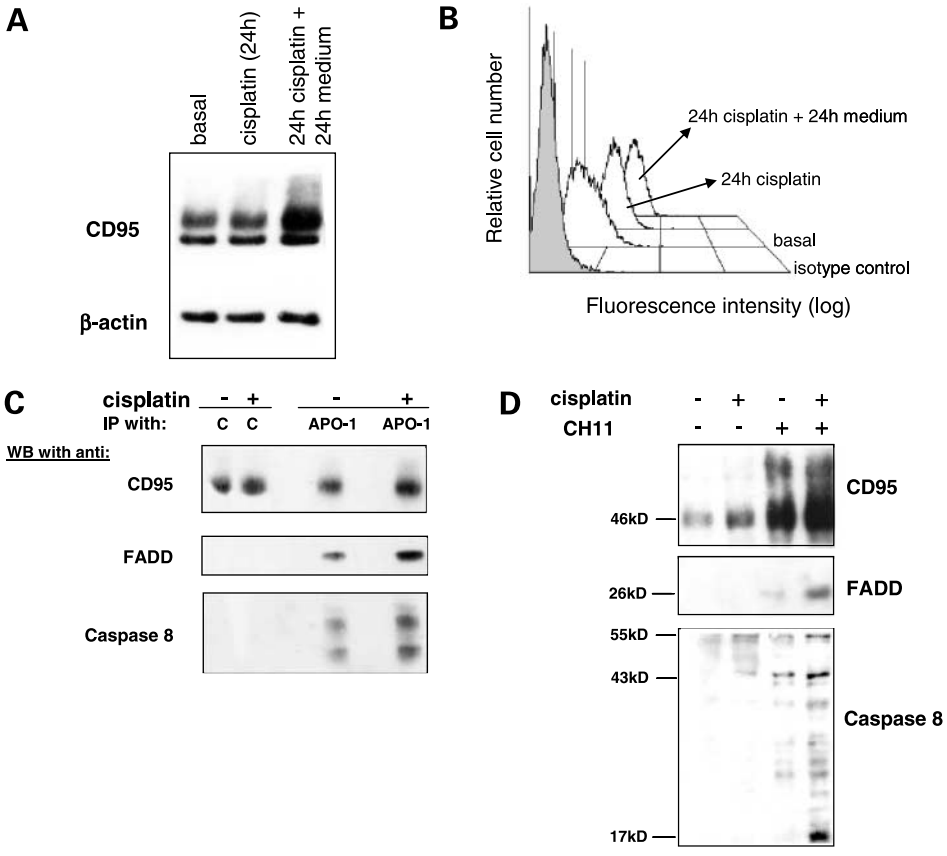

Figure 3. Subcytotoxic doses of cisplatin enhance CD95 expression and DISC recruitment in OVCAR3 cells. **A,** Western blot analysis for CD95 detection. Cells were lysed immediately after 24 h of treatment with cisplatin at the IC_{30} or after additional 24-h incubation in fresh medium. Untreated cells were used as control (*basal*). Blots were probed with rabbit anti-CD95 and anti–β-actin as loading controls. Representative of three experiments. **B,** flow cytometric analysis of CD95 cell-surface expression on OVCAR3 cells untreated or treated as described above and stained with FITC-anti-CD95 antibody (*open areas*) or with an isotype-matched control antibody (*gray-filled area*). *Arrows,* type of treatment. Results from a representative experiment of three. **C,** DISC immunoprecipitation from cells left untreated or exposed to cisplatin at the IC_{30} for 24 h, washed, and stimulated with anti-CD95 APO-1 (immunoglobulin G3) antibody for 30 min. Control cells (*C*) were lysed and then incubated with APO-1 and immunoprecipitated. Total cell lysates (400 μg) were immunoprecipitated with antimouse immunoglobulin G–coated magnetic beads and products were separated by 4% to 12% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-CD95 (C-20), anti-FADD, and anti–caspase-8 antibodies. Representative of three experiments. **D,** analysis of the Triton X-100–insoluble fraction of cells exposed to different treatments as indicated. Cell lysates were separated by 12% SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with antibodies directed to DISC components.

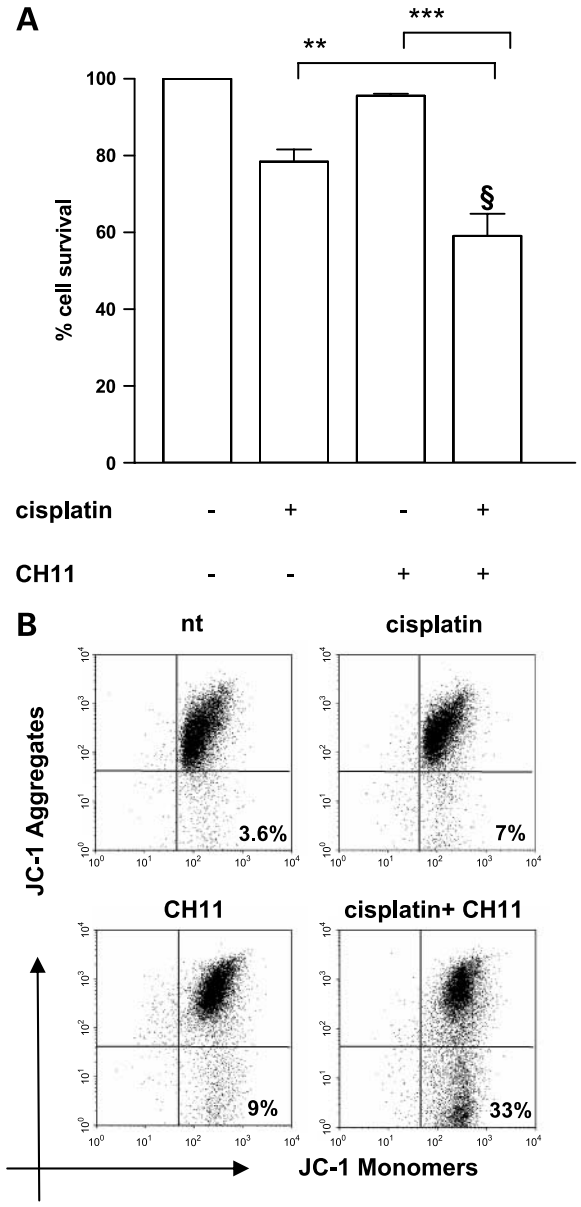

Figure 4. Cisplatin pretreatment increases sensitivity to CD95-mediated apoptosis in OVCAR3 cells. **A,** analysis of cell growth following treatment with 3 μmol/L cisplatin (IC_{30}) and/or with 300 ng/mL agonistic anti-CD95 antibody (CH11 clone) as indicated. *Columns,* mean of three independent experiments; *bars,* SD. ***, $P < 0.001$; **, $P = 0.007$ (unpaired *t* test). §, Drewinko index = 1.25 (calculated as described in Materials and Methods) indicates a synergistic effect of cisplatin and CH11 sequential treatment. **B,** analysis of mitochondrial membrane potential in cells treated as described above. At the end of treatments, cells were loaded with JC-1 cationic lipophilic dye and analyzed by flow cytometry.

improve DISC formation efficiency. The better recruitment of the adaptor molecule FADD eventually allowed an enhanced recruitment of caspase-8, which did not accumulate as procaspase but was further processed, as indicated by the presence of low molecular weight cleaved forms (*lane 4*).

Cisplatin Pretreatment Increases Sensitivity to CD95-Mediated Apoptosis in p53mu OVCAR3 Cells by Triggering Both Intrinsic and Extrinsic Apoptotic Signaling Pathways

Cell growth of OVCAR3 cells exposed to subcytotoxic doses of cisplatin (IC_{30}) or to anti-CD95 antibody remained essentially unchanged as compared with untreated cells [78 ± 3.2% and 95 ± 0.49%, respectively (mean ± SD); $n = 3$], whereas sequential exposure to subcytotoxic doses of cisplatin and anti-CD95 treatment led to a highly significant ($P < 0.001–0.007$) reduction in cell survival (59 ± 5.78% of cell growth; $n = 3$) as compared with cells untreated or exposed to single treatment. The combined treatment exerted a synergistic effect (Drewinko index = 1.25) according to Drewinko et al. (ref. 20; Fig. 4A).

Whereas neither cisplatin nor anti-CD95 antibody alone led to a decrease in mitochondrial membrane polarization, which was detectable only in 7.2 ± 2.9% (mean ± SD; $n = 3$) of single agent–treated cells, the combination of cisplatin and anti-CD95 antibody stimulated substantial membrane depolarization in 30 ± 4.2% ($n = 3$) of treated cells. A representative experiment is shown in Fig. 4B. Western blot

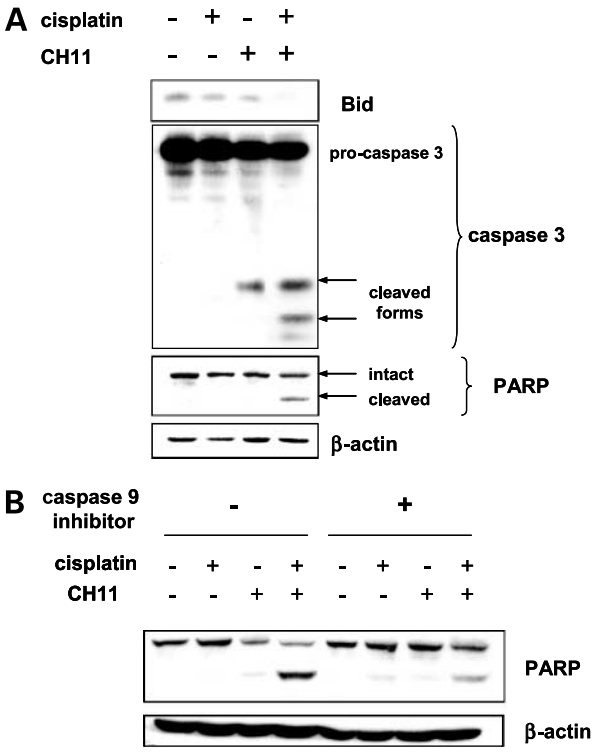

Figure 5. Apoptosis induced by combined treatment involves mitochondrial pathways in p53mu OVCAR3 cells. **A,** Western blot analysis of proteins in the extrinsic apoptotic pathway in OVCAR3 cells treated as indicated. Total cell lysates (40 μg) were separated by 12% SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with antibodies to Bid, caspase-3, and PARP. **B,** cells were treated with cisplatin and/or CH11, as described, in the presence or absence of a cell-permeable caspase-9 inhibitor. After treatment, 40 μg of total cell lysates were separated by 10% SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with anti-PARP and anti–β-actin (loading control) antibodies.

Table 1. Clinical and pathologic features of EOC patients

Patient ID	Age (y)	Histotype	Grade	Stage	p53 status	p53 sequence*	c-FLIP expression†
TM0408A	51	Serous	3	IIIC	Mut	Exon 6 $His^{193}Arg$ (CAT>CGT)	Neg
TM0311A	64	Serous	3	IV	Mut	Exon 8 $Val^{272}Met$ (GTG>ATG)	Neg
TM0502A	49	Serous	2	IIIC	Wt	Intron 8 donor GT>AT	Neg
TM0505A	74	Serous	2	IIIC	Wt	Exon 6 polymorphism codon 213 (CGA>CGG)	Pos
TM0509A	51	Serous	3	IIIC	Wt	Wt	Pos
TM0516A	85	Serous	3	IV	Mut	Exon 8 $Arg^{282}Gly$ (CGG>GGG)	Neg

*Molecular analysis of p53 status was done on genomic DNA extracted from each sample and screened as described (6).
†Evaluated by immunohistochemistry, essentially as described (6), on tumor samples obtained at the time of ascites collection.

analysis indicated disappearance of the intact form of Bid in the sequentially treated cells (Fig. 5A), supporting the involvement of the mitochondrial pathway. Caspase-3 activation, as confirmed by decreased expression of the caspase proform and the appearance of the respective cleavage products, was also consistently observed. Although partial caspase-3 activation was detected in cells treated with anti-CD95 antibody alone, full activation of the apoptotic cascade was observed only in cells exposed to the combined treatment as shown by the increase in the cleaved form of PARP enzyme, which is the major caspase-3 target. Densitometric analysis indicated that, in these cells, cleaved PARP normalized to total protein amount (β-actin ratio) represented 43%, as compared with 5% in cells exposed to anti-CD95 antibody alone (Fig. 5A).

The up-modulation of CD95 death receptor expression accompanied by an increased efficiency of DISC formation and caspase-8 processing, as well as the detection of Bid cleavage after the sequential treatment, suggested the initiation of an efficient apoptotic signaling. To examine the involvement of the mitochondrial pathway, experiments were carried out in which mitochondrial signaling was impaired by blocking caspase-9 activity. Thus, OVCAR3 cells were incubated with cisplatin and anti-CD95 antibody alone or in combination, in the presence or absence of the cell-permeable caspase-9 inhibitor z-LEHD-fmk, and total cell lysates were analyzed by Western blotting (Fig. 5B). In cells exposed to the combined treatment, the z-LEHD-fmk reagent partially inhibited PARP cleavage, with the cleaved form of PARP accounting for 29%, as compared with 67% in the absence of the inhibitor, thus confirming the activation of an efficient mitochondrial pathway in apoptotic signaling. The relative percentage of PARP cleavage was evaluated after normalization to total protein amount.

Cisplatin Pretreatment Induces Sensitization to CD95-Dependent Apoptosis in EOC Patient's Ascitic Cells

Table 1 summarizes the clinical and pathologic features of advanced-stage EOC patients whose ascitic fluids were used to test the therapeutic potential of cisplatin/anti-CD95 combination treatment versus that of either reagent alone. Tumor samples obtained at the time of ascites collection were also analyzed for c-$FLIP_L$ expression, considerably confirming the previously described inverse relationship with p53 status.

Two clinically relevant doses of cisplatin were used for these experiments: the higher (10 μmol/L) to test the optimal chemotherapeutic regimen and the lower dose (5 μmol/L) as the death receptor–dependent apoptosis-sensitizing agent. As in the case of OVCAR3 cells, exposure to the subcytotoxic cisplatin dose induced an increase in CD95 expression also on tumor cells derived from ascitic fluids. In Fig. 6A, an experiment referring to a p53mu ascites is shown. Furthermore, as shown in Fig. 6B, the apoptotic effects after treatment with anti-CD95 antibody, low-dose cisplatin, or high-dose cisplatin were only marginal, whereas the combined treatment determined a substantial increase in apoptosis. Due to the limited total cell number, PARP cleavage was used as a surrogate indicator of cell death to enable a biochemical quantification of the observed effect. The relative percentage of the cleaved form of PARP was evaluated after normalization to total protein amount (β-actin ratio); in all six samples, it was significantly higher ($P = 0.01$, unpaired t test) in EOC cells exposed to the combined versus single-agent treatment [51.5 ± 12.4% and 17.6 ± 4.9%, respectively (mean ± SD); Table 2]. The same analysis done on the subgroup of patients bearing a mutated p53 revealed 75.03 ± 14.3% of cleaved PARP after exposure to the cisplatin/anti-CD95 combination as compared with 26.32 ± 6.2% in cells exposed to either single agent ($P = 0.01$; Table 2; Fig. 7). Importantly, combined treatment in these samples showed a synergistic effect (Drewinko index = 1.66) in inducing cell death.

Discussion

Cancer treatment with chemotherapeutic agents causes cytotoxicity primarily by the induction of apoptosis. However, resistance to therapy remains an important clinical problem. Extensive interest has recently focused on defects in the apoptotic signaling pathway as the cause of treatment resistance, and identification of new therapeutic strategies to overcome apoptosis resistance is a major goal.

First-line chemotherapy with platinum and taxane compounds is the standard procedure for EOC. However, in many tumors, loss of p53 functional activity is associated with platinum resistance. Thus, we aimed to identify strategies able to overcome apoptosis resistance in EOC

cells, particularly in tumors bearing a p53 mutation. Our data show that the p53mu EOC OVCAR3 cell line, which is resistant to receptor-mediated apoptosis and relatively insensitive to cisplatin, is efficiently induced to CD95-mediated apoptosis after pretreatment with subcytotoxic but clinically relevant doses of cisplatin. These data are of particular relevance because the combination of subcytotoxic cisplatin doses and CD95 triggering synergized in inducing cell death, even in short-term cultured tumor cells derived from advanced-stage EOC patients' ascitic fluid.

A variety of inhibition mechanisms of receptor-mediated apoptosis have been identified thus far. In this context, we recently showed that c-FLIP$_L$ overexpression underlies apoptosis resistance in p53wt EOC and showed a significant inverse relationship between c-FLIP$_L$ expression and p53 mutation in case material from advanced-stage EOC patients (6), suggesting that c-FLIP–mediated inhibition might protect tumors where p53 mutation has not yet occurred. This mechanism is not involved in the resistance to receptor-mediated apoptosis in p53mu cell lines. We report here that in these p53mu cells, efficiency of DISC formation was poor and resulted in low-level Bid cleavage, which was not sufficient to trigger mitochondrial amplification of apoptotic signal. Cytotoxic drugs can activate CD95 signaling pathways through up-regulation of the receptor and/or ligand (26), and this activation may be an essential factor in the early phase of drug-induced cell death. In fact, we found that exposure to subcytotoxic but clinically relevant cisplatin concentrations consistently induced CD95 membrane expression and promoted recruitment of DISC components by relocalizing the CD95 receptor in membrane lipid rafts, thus allowing more efficient CD95 triggering and apoptotic signaling. The enhanced recruitment of DISC elements favored complete caspase-8 activation. Indeed, we did not observe procaspase-8 accumulation because it was processed in low molecular weight forms as it was recruited to the DISC. The partial reversion of apoptosis observed in the presence of caspase-9 inhibitor z-LEHD-fmk showed that in the case of combined treatment, apoptotic signaling proceeded through activation of an efficient mitochondrial pathway.

A similar CD95 trafficking to cell membrane due to chemotherapeutic agents has been also reported in colorectal cancer (27). In our experimental system, in spite of a consistent up-regulation following cisplatin exposure, CD95 expression level in p53mu OVCAR3 cells remained lower than the constitutive expression level detectable in p53wt OAW42 cells, and drug treatment alone was not sufficient to efficiently recruit DISC components. Nevertheless, we showed that, in the p53mu context, cisplatin pretreatment was necessary to complete caspase-8 processing because receptor triggering alone proved ineffective. Although in this cell line c-FLIP$_L$ expression is not relevant in apoptosis inhibition (6), we cannot rule out the

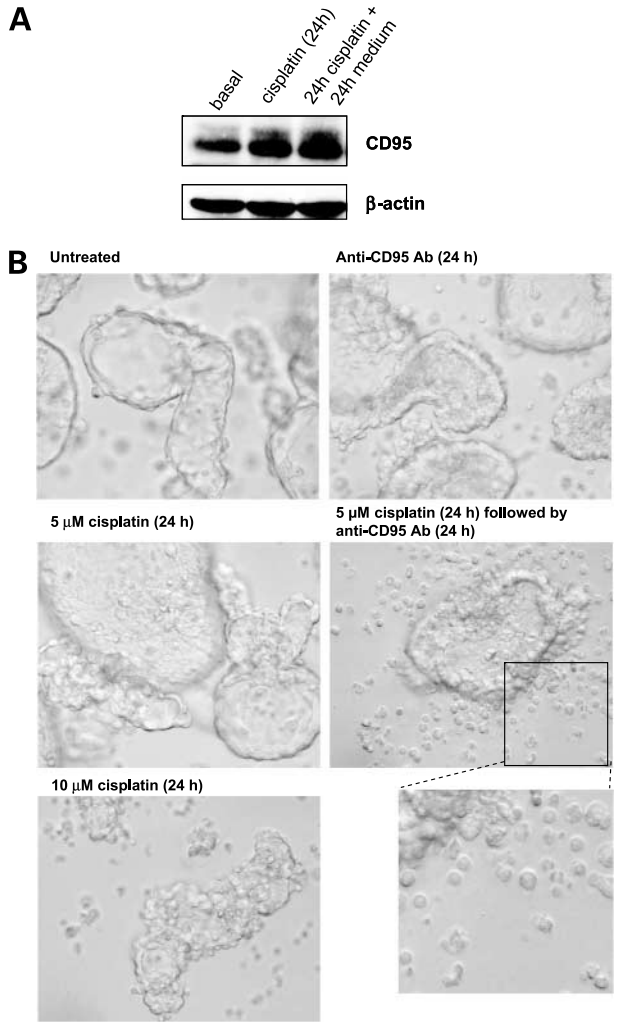

Figure 6. Cisplatin pretreatment increases CD95 expression and sensitivity to CD95-mediated apoptosis in tumor cell clumps from advanced EOC patients' ascitic fluid. **A,** Western blot analysis for CD95 detection on cells derived from patient TM0311A and treated as indicated. Untreated cells were used as control (*basal*). Blots were probed with rabbit anti-CD95 and anti–β-actin as loading controls. **B,** EOC cells from ascitic fluid of patient TM0505A (see Table 1) were treated as indicated. Original magnification, ×400. *Boxed area,* higher magnification of apoptotic cells.

Table 2. PARP cleavage in EOC samples

	All samples (n = 6)	P*	p53mu samples (n = 3)	P*
Untreated	3.75 ± 1.4	0.05	4.7 ± 2.4	0.04
Single treatment†	17.6 ± 4.9		26.32 ± 6.2	
Combined treatment‡	51.53 ± 12.4	0.01	75.03 ± 14.3	0.01

NOTE: Values represent PARP cleavage expressed as mean (±SD) percentage of total protein amount (β-actin-normalized). Densitometric analysis was done using ImageQuant software.

*$P \leq 0.05$ is considered as significant (unpaired t test).

†Data obtained with cisplatin or anti-CD95 antibody given alone. Levels of PARP cleavage in the two groups were not significantly different.

‡Data obtained after 24-h pretreatment with 5 μmol/L cisplatin followed by 24-h treatment with anti-CD95 antibody.

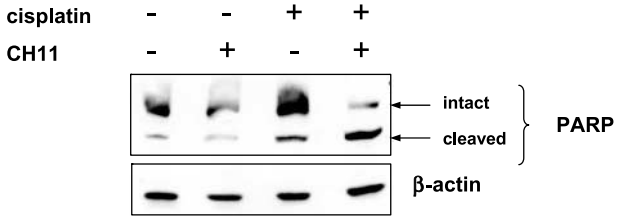

Figure 7. Cisplatin and anti-CD95 antibody combined treatment increases apoptosis in tumor cell clumps from advanced EOC patients' ascitic fluid. Western blot analysis for PARP cleavage in tumor cells derived from ascitic fluid of patient TM0516A. Tumor cells were treated as indicated and total cell lysates (40 μg) were separated by 10% SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with anti-PARP and anti–β-actin (loading control) antibodies.

possibility that besides the CD95 up-regulation, combination of the two treatments succeeded in DISC activation through a cisplatin action on other caspase-8 regulatory factor(s), eventually favoring apoptotic signaling. These findings are further supported by our previous CD95 transfection experiments where, in spite of an increase of CD95 receptor, we did not observe a proportional increase of CD95-mediated apoptosis.[5]

DNA-damaging drugs like cisplatin, at concentrations comparable to those present in sera of patients during therapy, are known to induce CD95 as well as CD95L expression in hepatoma cells, gastric, colon, and breast cancer cell lines carrying wt p53 (13). In these experimental models, whereas induction of CD95L occurs independently of p53 status, up-regulation of CD95 receptor seems to be restricted to p53wt cells because p53 directly transactivates the *CD95* gene (13). To explain the apparent discrepancy of our results, it should be noted that, recently, other proteins belonging to p53 family (i.e., p63 and p73) were shown to participate in p53-mediated DNA damage responses (28) and that p53-p73 cross-talk has been documented in EOC (29). Moreover, Muller et al. (15) and Gressner et al. (16) recently showed that the *CD95* gene in hepatoma cells is a direct transcriptional target of both TAp63α and TAp73β, which act through the p53 binding site in the first intron of the *CD95* gene. Accordingly, we observed a slight increase of CD95 mRNA in OVCAR3 cells after cisplatin exposure.[5] Thus, it seems possible that TAp63α and TAp73β may be able to sensitize cancer cells to chemotherapy in a functionally inactive p53 context (30).

Irrespective of the specific mechanism underlying cross-talk between extrinsic and intrinsic apoptotic pathways, the effectiveness of a combined treatment is evident also in short-term cultured ovarian tumor cells from ascitic fluid of advanced-stage EOC patients. Our analysis of the response to the combined treatment revealed a significant improvement in apoptosis signaling in all tested cases, independently of p53 functional status. A therapeutic advantage could be obtained mainly in p53-inactivated EOC cells, in which combination treatment has a synergistic effect and significantly increased the killing, further supporting the efficacy of this therapeutic strategy.

After initial attempts to develop therapeutic anti-CD95 antibodies were confounded by severe hepatic toxicity in murine models (31), interest has grown in targeting the death receptor tumor necrosis factor–related apoptosis-inducing ligand and other members of the tumor necrosis factor family shown to exert antitumor effects without serious side effects in xenograft models (32, 33). However, a novel agonistic anti-CD95 antibody that lacks hepatic toxicity has recently been developed (27, 34), suggesting the feasibility of obtaining clinically useful CD95-triggering antibodies. Because several different cytotoxic drugs are able to induce CD95 membrane expression (35), combined use of chemotherapeutic agents with anti-CD95 antibodies or death receptor ligand holds promise as an anticancer strategy.

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