CD95-Mediated Apoptosis Is Impaired at Receptor Level by Cellular FLICE-Inhibitory Protein (Long Form) in Wild-Type p53 Human Ovarian Carcinoma

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ABSTRACT

Purpose: Ovarian carcinoma is a highly lethal malignancy that often becomes resistant to chemotherapy. Alterations in apoptotic signals and p53 status contribute to drug resistance, and CD95-mediated apoptosis is also deficient in resistant cells. We analyzed the mechanism of resistance to CD95-mediated apoptosis in ovarian carcinoma cell lines differing in p53 status.

Experimental Design: CD95-mediated apoptosis was induced by agonistic anti-CD95 antibody, and the apoptotic cascade was monitored with biochemical and functional assays.

Results: CD95-mediated apoptosis was blocked in human ovarian cancer cells. In cell lines with wild-type p53, treatment with the protein synthesis inhibitor cycloheximide (CHX) together with anti-CD95 overcame the resistance, suggesting the presence of a labile inhibiting protein. Indeed, the labile protein cellular FLICE-inhibitory protein long form (c-FLIPL) was found to block caspase-8 recruitment to the death-inducing signaling complex (DISC), and sensitization of cells by CHX was due to c-FLIPL down-modulation at the DISC level. Down-regulation of c-FLIPL with antisense oligonucleotides increased CD95-mediated apoptosis as in cells sensitized by CHX, demonstrating the direct involvement of c-FLIPL in apoptosis resistance. Removal of c-FLIPL block at DISC level allowed full activation of the mitochondrial pathway and, eventually, apoptosis in wild-type p53 cells, whereas in cells with mutated p53, c-FLIPL involvement in CD95-mediated apoptosis resistance appeared to be irrelevant. Immunohistochemical analysis of an ovarian tumor tissue array revealed c-FLIPL expression in samples with no p53 accumulation (P = 0.034), and a significant (P = 0.037) inverse relationship between c-FLIPL and p53 expression levels was also observed in 27 epithelial ovarian cancer specimens with known p53 status.

Conclusion: The inhibitory protein c-FLIPL is involved in resistance to CD95-mediated apoptosis in ovarian carcinoma cells with wild-type p53.

INTRODUCTION

Epithelial ovarian cancer is the leading cause of death in women with gynecological malignancies (1). Primary cytoreductive surgery followed by platinum-based chemotherapy is the standard treatment for this cancer. After a first high response rate, the patient usually relapses, and the tumor becomes resistant to chemotherapy. Such resistance has been associated with decreased susceptibility to apoptosis (2, 3), with p53 status as one of the major determinants. p53 inhibits progression of stressed cells through the cell cycle and even induces apoptosis as a mechanism to contain the damage and protect the organism (4). The proapoptotic activity of p53 is mediated through the mitochondrial pathway by activation of Bcl-2 family members such as Bax. Thus, the p53 protein provides a critical brake on tumor development consistent with its mutated states and, thus, inactivation in the majority of cancers.

Apoptosis can be also triggered by ligand-dependent signaling involving the CD95 (Fas/APO-1) molecule (5, 6). The importance of the CD95 receptor pathway in drug-induced apoptosis has been recognized (7–9), and the deficient activation of this pathway has been implicated in drug resistance in renal cancer (10). Indeed, impaired or disrupted apoptotic pathways are involved in tumor progression and in resistance to therapy (3, 11, 12). At physiological conditions, the interaction of CD95 with its natural ligand (CD95L) induces apoptosis of CD95-expressing cells (13, 14). Ligation of CD95 induces its trimerization and recruitment of the adapter molecule Fas-associated death domain (FADD) and of procaspase-8 to form the death-inducing signaling complex (DISC; Ref.15). Procaspase-8 becomes activated and, in turn, triggers activation of the “effector” caspases 3, 6, and 7. These enzymes cleave several cellular substrates, ultimately leading to cell death. On the basis of the strength of caspase-8 activation at the DISC level, two distinct cell types can be identified (16). In type I cells, CD95 triggering leads to strong caspase-8 activation in the DISC, leading to direct activation of downstream effector caspases. In type II
cells, the DISC is not efficiently formed, andactivation of effector caspases depends on the mitochondrial pathway (16). This cross-talk between the death receptor and mitochondrial pathways is provided by BID, a proapoptotic Bcl-2 family member. Caspase-8-mediated cleavage of BID greatly increases its proapoptotic activity. The truncated form, tBID, translocates to the mitochondria and promotes oligomerization of Bax, which allows the release of cytochrome c from the mitochondria into the cytosol (17, 18). In the presence of ATP, cytochrome c binds to apoptotic protease-activating factor-1, which then oligomerizes and binds procaspase-9 to form the multimeric apoptosome complex. This complex activates caspase-9 and eventually caspase-3 (19). Tumor cells, however, can resist CD95-mediated apoptosis despite the expression of CD95 on their surface.

Inactivation of p53 can induce apoptosis resistance, mainly by blocking the mitochondrial pathway. On the other hand, CD95 signal transduction can be inhibited directly at the receptor level by molecules, such as cellular FLICE-inhibitory protein (c-FLIP), interacting with the DISC formation (20, 21). The long isoform of c-FLIP (FLIPL) is a potent inhibitor of cell death and may be processed at the DISC into a p43 intermediate product that forms a complex with cleaved caspase-8. This complex, which remains at the receptor level, inhibits the new recruitment and processing of caspase-8, thus blocking the death signal (20). Further inhibition of CD95 signal transduction might occur along the mitochondrial pathway (16, 22) through the action of Bcl-2 family members such as Bcl-2 itself and Bcl-xL (22). Defects in apoptotic protease-activating factor-1, and therefore in apoptosis formation, have been shown to block the signals downstream of mitochondria in a cell-free system (23, 24).

Here we show that in malignant human ovarian cells with wild-type p53 and with a functionally active mitochondrial pathway, CD95-mediated apoptosis is blocked at receptor level. We observed relevant recruitment of c-FLIPL to the DISC and reduced caspase-8 activation. Down-regulation of c-FLIPL by antisense oligonucleotides increased CD95-mediated apoptosis only in wild-type p53 cells, demonstrating the direct involvement of c-FLIPL in apoptosis resistance. Immunohistochemical and biochemical analyses of tissue samples from ovarian cancer patients revealed c-FLIPL overexpression in tumors with wild-type p53, whereas in tumors with mutated p53, c-FLIPL was down-modulated. Thus, c-FLIPL may act to protect tumors in which mutation of p53 has not yet occurred.

MATERIALS AND METHODS

Cell Lines. The following human serous ovarian carcinoma cell lines were used: IGROV1 (a gift from J. Bénard, Institute Gustave Roussy, Villejuif, France); OVCAR3 and SKOV3 (American Type Culture Collection, Manassas, VA); OAW42 (kindly provided by A. Ullrich, Max Planck Institute of Biochemistry, Martinsried, Germany); OVCA432 (kindly provided by R. Knapp, Dana-Farber Institute, Boston, MA); and INT.Ov1 and INT.Ov2 (generated in our Institute; Ref. 25). IGROV1 (26) and OAW42 (27) carry wild-type p53, whereas OVCAR3, SKOV3, and OVCA432 are p53-mutated. The p53 status in the INT.Ov1 and INT.Ov2 lines was not yet determined.

Cell lines were cultured in RPMI 1640 except for OAW42, which was maintained in MEM supplemented with 10% fetal calf serum, 2 mM glutamine, and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO2 at 37°C.

Anti-CD95-Mediated Cytotoxicity. Cells were plated in triplicate at 4 × 10⁴ cells/well in flat-bottomed 96-well microtiter plates and allowed to adhere. After overnight incubation at 37°C, we added to each well 300 ng/ml agonistic IgM anti-CD95 monoclonal antibody (mAb; clone CH-I1; MBL Co., LTD, Nagoya, Japan) in the presence or absence of 1 μg/ml cycloheximide (CHX, Sigma Chemical Co., St. Louis, MO) and incubated the plates at 37°C for different times. Cell viability was assessed by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay as described previously (28). The cytotoxicity in cells treated with anti-CD95 was compared with that in cells incubated with medium alone (100% viability) and with cells incubated with irrelevant isotype-matched mAb in the presence or absence of CHX.

DNA Fragmentation Analysis. Qualitative DNA fragmentation was analyzed as described previously (29). Briefly, 7 h after treatment with anti-CD95 or anti-CD95 plus CHX, both adherent and floating cells were harvested, pelleted, washed once, resuspended in 200 μl of lysis buffer [10 mM Tris(HCl) (pH 8.0), 10 mM EDTA, 0.5% Triton X-100] and kept on ice for 5 min. After centrifugation at 10,000 × g, the supernatant was collected and treated with 100 μg/ml RNase A (Boehringer Mannheim, Mannheim, Germany) overnight at 37°C. SDS and proteinase K (Sigma) were added to final concentrations of 0.5% and 200 μg/ml, respectively, and extracts were incubated for 2 h at 50°C. Low-molecular-weight DNA was extracted twice in phenol and once in phenol–chloroform, and ethanol-precipitated. DNA was resuspended in Tris-EDTA buffer and loaded on a 1.5% ethidium bromide-stained agarose gel.

Western Blotting and Immunoprecipitation. For Western blot analysis, cells were washed twice in cold PBS and lysed for 15 min on ice in lysis buffer [10 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.02% Na3VO4, 1% NP40, 0.1% SDS]. Cell lysates were centrifuged (13,000 rpm for 15 min at 4°C), the supernatant was recovered and the protein concentration was determined by bicinchoninic acid protein assay (Pierce, Rockford, IL). Tissue lysates were obtained from six ovarian carcinoma specimens treated as follows: for each specimen, 100% viability) and with cells incubated with irrelevant isotype-matched mAb in the presence or absence of CHX.

the case of tumor specimens) were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Hybond C-Super; Amersham). Membranes were saturated in Blotto (5% nonfat dry milk, 0.1% Tween 20 in PBS) and incubated overnight at 4°C with the recommended concentrations of primary antibodies: mouse mAb to FADD and to poly(ADP-ribose) polymerase-1 (PARP-1); rabbit polyclonal antibody to caspase-3 (PharMingen, BD Biosciences); mouse mAb 1C12 to caspase-8 (Cell Signaling Technology); mouse mAb DO7 to p53 (Novocastra Laboratories Ltd., Newcastle-upon-Tyne, United Kingdom); rabbit polyclonal antibody to BID (Cell Signaling Technology); rabbit polyclonal antibody to FLIP<sub>L</sub> (H-202; Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit polyclonal antibody to CD95 (Santa Cruz Biotechnology). The relevant secondary peroxidase-labeled antibodies (Amersham) diluted in Blotto were added for 1 h at room temperature. Reactions were visualized with the enhanced chemiluminescence technique (Amersham).

For analysis of DISC components, cells were incubated for 4 h at 37°C with 2 μg/ml murine anti-c-CD95 mAb APO-1 (Alexis Biochemicals) and lysed in lysis buffer [30 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride]. Control cells were incubated with the APO-1 mAb after lysis. The CD95 receptor and the associated proteins in the DISC complex were immunoprecipitated overnight at 4°C with goat antimouse IgG Dynabeads (Dynal). Beads were washed five times with lysis buffer, resuspended in SDS-PAGE sample buffer, and heated for 3 min at 95°C. Immunoprecipitates were separated by 10% SDS-PAGE and immunoblotted with anti-c-CD95, anti-FADD, anti-caspase-8, and anti-FLIP<sub>L</sub> antibodies.

**Transfection with Antibiotic Oligonucleotides.** Phosphorothioate single-stranded antisense oligonucleotides directed against the human FLIP translation initiation codon (5′-TCTTTCCAGCTGGGCAAT-3′) and the control nonsense oligonucleotide (5′-GACTTACGACGACTTCCAT-3′; Ref. 30) were purchased from MWG-Biotech AG (Ebersberg, Germany). OAW42 and OVCAR3 cells grown to 60% confluence were incubated with the recommended concentrations of primary antibodies: mouse mAb to FADD and to poly(ADP-ribose) polymerase-1 (PARP-1); rabbit polyclonal antibody to BID (Cell Signaling Technology); anti-caspase-8, and anti-FLIP antibodies.

**Fluorometric Assay for Caspase Activity.** Caspase activity from cytosolic extracts (see below) or lysates of cells treated as indicated was measured by spectrophotofluorometric assay using synthetic fluorogenic substrates DEVD–D-aminoo-4-trifluoroacetyl-l-arginylcarboxyfluorescein (DEVD-AFC; for caspase-3) and IETD-AFC (for caspase-8; both from PharMingen). Cleavage specificity was assessed by performing the assay in the presence of the inhibitors DEVD-CHO and IETD-CHO (PharMingen), respectively. After the indicated treatments, cells were washed and lysed; cytosolic extracts were collected by centrifugation and assayed for caspase activity by incubation with the fluorogenic substrates for 1 h at 37°C. Fluorescence was detected with a fluorometer equipped with a 485 nm excitation and a 505 nm emission filter. Relative AFC fluorescence was normalized to lysate protein concentrations.

**Subcellular Fractionation and Cytochrome c Release.** Cells treated as indicated were washed twice with PBS, resuspended in mitochondrial buffer [70 mM Tris (pH 7.4), 250 mM sucrose, 1 mM EDTA], and kept on ice for 10 min. An equal volume of MES buffer (19.8 mM EGTA, 19.8 mM EDTA, 250 mM mannitol, 19.8 mM MES (pH 7.4)] containing 2 mg/ml digitonin and protease inhibitor cocktail was added until cells were permeabilized (checked by trypan blue exclusion). Nuclei and cellular debris was removed by centrifugation at 900 × g for 5 min. The supernatant was further centrifuged at 20,000 × g for 15 min to collect the mitochondria-enriched fraction (pellet), which was resuspended in 100 μl of mitochondrial buffer, and the cytosolic fraction (supernatant). Proteins (40 μg) were separated by 12% SDS-PAGE, blotted, and probed with mouse mAb to cytochrome c (PharMingen, BD Biosciences).

**Immunohistochemical Detection of c-FLIP<sub>L</sub> and p53.** Immunohistochemical detection of c-FLIP<sub>L</sub> and p53 was performed on a tissue array slide constructed with paraffin sections from 55 ovarian specimens of different histotypes (SuperBioChips Laboratories; see Table 1) and on formalin-fixed, paraffin-embedded sections from 27 epithelial ovarian cancer patients with known p53 status (12 wild-type and 15 mutated). After xylene deparaffinization and alcohol rehydration, sections were subjected to antigen retrieval in 10 mM citrate buffer (pH 6) in a pressure cooker. Endogenous peroxidase was quenched by incubating the slide with 3% H<sub>2</sub>O<sub>2</sub> for 6 min. After being washed with PBS, slides were incubated in serum-containing blocking solution for 30 min. Primary antibody goat anti-FLIP<sub>L</sub> C-19 (Santa Cruz Biotechnology) at 1:100 dilution was applied for 2 h at room temperature. C-19 is a goat polyclonal antibody raised against a peptide mapping to the carboxyl terminus of human c-FLIP<sub>L</sub> and does not cross-react with FLIP<sub>S</sub> or other members of the caspase family. Slides were then washed with PBS and incubated for 30 min at room temperature with 1:200 biotin-conjugated goat secondary antibody (Vestastain Elite ABC Kit; Vector Lab). The p53 immunoreactivity was measured as described previously (31) with use of the mouse anti-p53 mAb DO7 (Novocastra Laboratories). Slides were washed with PBS and incubated with the avidin-biotin complex (Vector Lab) for 30 min. The peroxidase reaction was developed with 3,3-diaminobenzidine, and sections were counterstained with hematoxylin. Slides incubated with secondary antibodies alone provided negative controls; a control with hematoxylin and eosin was also prepared.

**Molecular Analysis of p53.** Methylenear-blue-stained sections from formalin-fixed, paraffin-embedded tissues were carefully microdissected under the microscope to obtain malignant tissues. Genomic DNA was extracted from the tissues and screened by double-gradient-denaturing gradient gel electrophoresis for the presence of TP53 mutations in the most frequently affected exons (exons 5–8) of the gene as described previously (32). Briefly, a two-step PCR protocol was carried out using as controls a cell line carrying a wild-type TP53 and a cell line carrying a known TP53 mutation.
were subjected to automated DNA sequencing (ABIprism 377; Applied Biosystems) and analyzed with Sequencing Analysis and Sequence Navigator software (ABI Prism). Each sequence reaction was performed at least twice, with separate amplifications analyzed, and the detected mutations were confirmed in the sequence as sense and antisense strands.

RESULTS

Resistance of Ovarian Cancer Cells to CD95-Mediated Apoptosis Can Be Overcome by CHX Treatment. The ability of an agonistic anti-CD95 (CH-11) mAb to induce apoptosis in different ovarian carcinoma cell lines was compared with that of an isotype-matched control antibody at 24 h. In repeated experiments, none of the ovarian carcinoma cell lines tested (Fig. IA, open columns) showed a level of sensitivity to CD95-mediated apoptosis exceeding 30% cytotoxicity. The same results were obtained with sCD95L. Increasing the antibody concentration did not lead to increased cytotoxicity (data not shown). Increasing the incubation time with agonistic anti-CD95 antibody significantly increased apoptosis in INT-Ov1 cells at both 48 and 72 h (61/11006 12.3% and 72/11006 8%, respectively, as evaluated by MTT assay). Increased apoptosis was

![Image](https://example.com/image.png)

Table 1 Immunohistochemical detection of c-FLIPₐ in ovarian tissue array

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<th>Moderate/Intense†</th>
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* Diffuse weak or focal intense staining.
† Moderate or intense staining of >80% of the tissue section.
‡ Focal staining of epithelial cells only.

Fig. 1 Cycloheximide (CHX) in combination with anti-CD95 antibody can overcomes resistance to CD95-mediated apoptosis. A, CD95-mediated apoptosis in ovarian carcinoma cell lines in the presence or absence of CHX. Cells were incubated for 24 h with 300 ng/ml agonistic anti-CD95 antibody CH-11 or isotype-matched control antibody in the presence (Lanes 2 and 6) or absence (Lanes 1 and 5) of 1 µg/ml CHX, a protein synthesis inhibitor. Cell viability was measured by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data represent mean percentage cytotoxicity (±SD; bars) of 3–10 independent experiments with anti-CD95 CH-11 alone (□), isotype-matched control antibody plus CHX (■), and CH-11 plus CHX (▲). The p53 status of the cell lines is reported. wt, wild type; n.d., not done. B, DNA fragmentation induced by anti-CD95 treatment. Ovarian carcinoma cell lines OAW42 and INT-Ov1 were treated for 7 h with anti-CD95 antibody CH-11 (lanes 2 and 6), CHX (lanes 4 and 8), or both (lanes 3 and 7) or left untreated (lanes 1 and 5), and DNA was extracted and analyzed for the presence of the typical ladder in an ethidium bromide-stained agarose gel.
also seen in OAW42 and IGROV1 cells, but only after 72 h of incubation. Because CD95-mediated apoptosis is a relatively rapid mechanism, these results suggest that most of the ovarian carcinoma cells tested are resistant to CD95 triggering and that, with the exception of INT-Ov1, very long incubation times (72 h) are needed to achieve cytotoxicity levels never exceeding 40%. On the other hand, CHX, which has been shown to increase CD95-mediated apoptosis (33), used in combination with anti-CD95 antibody CH-11 greatly enhanced the level of apoptosis observed in the OAW42 cells and IGROV1 cells bearing wild-type p53 as well as in INT-Ov1 and INT.Ov2 cells of unknown p53 status, but not in the remaining cell lines bearing mutated p53 (Fig. 1A). DNA fragmentation in OAW42 and INT.Ov1 cells after incubation with CH-11 plus CHX (Fig. 1B, Lanes 3 and 7, respectively) confirmed the apoptotic nature of the cytotoxicity observed in the MTT assay. DNA fragmentation after incubation in the presence of CH-11 alone was only partial (Fig. 1B, Lanes 2 and 6, respectively). The induction of CD95-mediated apoptosis in cells treated with the protein synthesis inhibitor suggests the presence of labile proteins able to block the CD95 signal.

CHX Treatment Increases Caspase Activation and PARP Cleavage in Cells with Wild-Type p53. Cytofluorimetric analysis in more than 30 independent evaluations of CD95 expression indicated mean fluorescence intensities (±SD) of 40.78 (±19.1) for OAW42 and 30.44 (±17.3) for OVCAR3 cells with a negative control of value of 6.9 (±2.85). Thus, CD95 expression in these two cell lines did not differ significantly. We selected the CHX-inducible wild-type p53 carcinoma cell line OAW42 and the noninducible mutated p53 line OVCAR-3 for analysis of the apoptotic cascade, based on their relatively similar levels of CD95 expression and their p53 status. Western blot analysis of the proximal procaspase-8 and the effector procaspase-3 revealed a clear disappearance of bands corresponding to procaspase-8 in inducible OAW42 cells after 24 h of cotreatment with CH-11 and CHX (Fig. 2A); the p43/41 intermediate cleavage products were also evident after treatment for 24 h with CH-11 alone. Treatment with antibody alone caused a decrease of procaspase-3 and the parallel appearance of the p89 cleavage product of PARP-1, a substrate of caspase-3. Maximum PARP cleavage never exceeded 30% of the total protein amount. After 24 h of cotreatment with CH-11 and CHX, procaspase-3 completely disappeared and caspase-3-activated p17 became evident. At this time point, maximum PARP cleavage was observed (cleaved form represented 60% of the total amount of protein), consistent with the level of apoptosis measured by MTT assay. Analysis of the noninducible cell line OVCAR3 indicated a similar appearance of the p43/41 procaspase-8 intermediate, although procaspase-8 never disappeared completely. The involvement of procaspase-3 was less evident, and no active p17 was detected; moreover, no increase in PARP cleavage was detected after any treatment, consistent with the lack of apoptosis determined by MTT assay. CHX alone had no effect on either cell lines (Fig. 2A).

Because the fragments corresponding to the cleaved forms of caspase-3 were barely detectable by Western blotting, the activity of caspase-3 after CD95 cross-linking was tested in cellular lysates by monitoring its ability to cleave the fluorogenic substrate DEVD-AFC. Consistent with the biochemical data (Fig. 2A), caspase-3 activation after CD95 triggering was detected only in OAW42 cells, where it was further increased by the addition of CHX (Fig. 2B).

DISC Formation Is Inefficient and c-FLIP{sub}L Is Recruited to the Complex in Ovarian Cells. In many cell lines, the first detectable event during CD95-mediated apoptosis is caspase-8 activation, which can occur as early as 5 s after receptor cross-linking (34) and usually takes place at the DISC level. Caspase-8 activation in ovarian cells was measured based...
on the ability of cytosolic preparations of anti-CD95-treated cells to cleave the IETD-AFC caspase-8-specific fluorogenic substrate. Analysis at different time points after receptor cross-linking revealed cleavage of the fluorogenic substrate only with the cytosolic extract of OAW42 cells and only after at least 4 h incubation with CH-11 (data not shown). This incubation time was then used for analysis of DISC formation.

To examine DISC formation, lysates from anti-CD95 APO-1 antibody-treated or untreated cells were immunoprecipitated with antiimmunoglobulin IgG-coated magnetic beads, resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed for all components of the DISC complex. The CD95 molecule was evident in the immunoprecipitates of all cell lines (shown in Lanes 2 for OAW42 and OVCAR3 in Fig. 3A) and in the controls where APO-1 was added after cell solubilization (Fig. 3A, Lanes 1). The adapter molecule FADD was clearly immunoprecipitated in OAW42 cells but barely detectable in OVCAR3 cells, although all of the ovarian cell lines tested showed comparable levels of this molecule in total cell lysates (data not shown). The two p55 isoforms and the p43 cleavage product of caspase-8 were detectable only at the DISC of OAW42 cells after long exposure of the filter. These data indicate that DISC formation in all ovarian cells is inefficient and suggest the need for mitochondrial involvement.

Decreased caspase-8 activation at the DISC level raised the possibility of the presence of an inhibitor protein such c-FLIP. The p43 cleavage product of c-FLIPp was present at the DISC level of all tested ovarian cancer cell lines, particularly in OAW42 (Fig. 3A), where the extent of recruitment was consistent with an inhibitory activity of this molecule. By contrast, recruitment at the OVCAR3 DISC was minimal and probably not sufficient for inhibitory activity. No evidence of recruitment of the short form of c-FLIP was observed either at the DISC or in total cell lysates despite the use of an antibody that recognizes both c-FLIP isoforms (data not shown). On the basis of the strong enhancement of CD95-induced apoptosis in OAW42 cells by the protein synthesis inhibitor CHX (Figs. 1 and 2), we tested whether this effect might rest in a down-regulation of the labile protein c-FLIP p43, leading to a reduced availability of this molecule for recruitment at the DISC. In OAW42 cells pretreated with CHX for 24 h before CD95 triggering and immunoprecipitation with APO-1 antibody, FADD recruitment to the DISC did not differ, caspase-8 recruitment improved, and levels of c-FLIP p43 cleavage product decreased dramatically compared with untreated cells (Fig. 3B). As expected, apoptosis (measured by MTT assay) increased significantly, from 13% to 33%, in CHX-pretreated cells. These data provide a molecular mechanism for the effect of CHX in CD95-treated OAW42 cells, i.e., protein synthesis inhibition reduces the amount of DISC-bound labile protein c-FLIP p43, ultimately leading to enhanced sensitivity to CD95-triggered cell death.

In OVCAR3 cells, the level of c-FLIP p55 recruitment to the DISC can be considered basal because it was essentially unchanged after CHX treatment (Fig. 3B), consistent with the resistance of these cells to CD95-mediated apoptosis even in the presence of CHX.

**Down-regulation of c-FLIP with Antisense Oligonucleotides Restores CD95 Sensitivity in p53 Wild-Type OAW42 Cells.** To confirm the functional relevance of c-FLIP p down-modulation in OAW42 sensitization to CD95-mediated apoptosis, we transfected OAW42 and OVCAR3 cells with antisense oligonucleotides to c-FLIP p and, as a control, with nonsense oligonucleotide. Transfection with c-FLIP p allele-specific oligonucleotides induced 60–70% down-modulation of the molecule in both cell lines (Fig. 4A). The effects of c-FLIP p down-modulation on CD95-mediated apoptosis after transfection with allele-specific oligonucleotides were evaluated biochemically as increased PARP cleavage after treatment with CH-11 for 24 h because such cleavage appears to represent an end point of the apoptotic process and because, in repeated experiments, PARP

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**Fig. 3** Defective death-inducing signaling complex (DISC) formation and cellular FLICE-inhibitory protein long form (c-FLIP L) recruitment in ovarian cancer cells. A, biochemical analysis of DISC formation. Cells were treated with 2 μg/mL CHX (IgG3) antibody for 4 h, lysed, and immunoprecipitated (IP). Control cells (C) were lysed and then incubated with APO-1 and immunoprecipitated. Total cell lysates (500 μg) were immunoprecipitated with antiimmunoglobulin IgG-coated magnetic beads. Immunoprecipitates were separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted (WB) with anti-CD95 (C-20), anti-Fas-associating death domain (FADD), anti-caspase-8, and anti-c-FLIP antibodies. Arrows indicate migration positions. B, treatment with cycloheximide (CHX) reduces the amount of DISC-bound c-FLIP p. Before CD95 cross-linking by APO-1, cells were treated with 1 μg/ml CHX for 24 h or left untreated. Immunoprecipitation (IP) was performed as described above. Immunoprecipitates were immunoblotted (WB) with anti-FADD, anti-caspase-8, and anti-c-FLIP antibodies. A long filter exposure was necessary for caspase-8 detection. Data are from a representative experiment of three performed with superimposable results.
cleavage levels were entirely consistent with percentage of cytotoxicity indicated in MTT assays (see Fig. 2). In OAW42 cells, down-modulation of c-FLIP<sub>L</sub> led to a significant increase in PARP cleavage (Fig. 4B). After normalization to total protein amount, cleaved PARP represented 43% compared with 20% and 14% in untreated or nonsense oligonucleotide-treated cells, respectively, reaching levels comparable to those observed after cotreatment with CH-11 and CHX (see Fig. 2). In OVCAR3 cells, down-modulation of c-FLIP<sub>L</sub> led to no detectable change in PARP cleavage (Fig. 4B). Thus, c-FLIP<sub>L</sub> plays a role in resistance to CD95-mediated apoptosis in cells expressing high levels of this inhibitory molecule.

**Restoration of Receptor Signaling Increases Cytochrome c Mitochondrial Release in OAW42 Cells.** The low level of caspase-8 recruited to the DISC after receptor crosslinking (see Fig. 3A) suggested that downstream apoptotic events are mediated by caspase-8 through induction of mitochondrial damage, as in type II cells (16). The specific proximal substrate of caspase-8 is BID. Indeed, CH-11 treatment of OAW42 cells induced partial cleavage of BID, evaluated as a decrease in the intact form of the molecule (Fig. 5). Cotreatment with CH-11 and CHX, responsible for down-modulation of c-FLIP<sub>L</sub> at the DISC level, led to a total disappearance of the molecule, indicating full activation of the mitochondrial pathway through tBID, which mediates release of cytochrome <i>c</i> from mitochondria. CH-11-treated cells evidenced a partial increase in cytochrome <i>c</i> release into the cytosolic fraction, concomitant with a decrease in the mitochondria-enriched fraction. When CHX was added to the assay, a complete shift of cytochrome <i>c</i> from mitochondria-enriched fraction to the cytosol was detected, further indicating full activation of the mitochondrial pathway.

These data support the notion that the impairment of CD95-mediated apoptosis in cells such as OAW42, which bear wild-type p53 and thus have a functional mitochondrial apoptotic pathway, is localized at the receptor level and is due to the labile protein c-FLIP<sub>L</sub>. In fact, blocking of this molecule by a protein synthesis inhibitor or antisense oligonucleotides led to activation of the mitochondrial pathway and, eventually, to cell death.

**c-FLIP<sub>L</sub> Is Expressed in Ovarian Cancer Tissues.** Immunohistochemical analysis of a tissue array containing 55 ovarian sections of different tumors revealed c-FLIP<sub>L</sub> expression in almost 50% of tumors of epithelial origin with variable intensity (Table 1). A focal subnuclear cytoplasmic staining was observed in epithelial cells surrounded by stromal tissue in endometrioid adenocanthoepithelioma and in borderline and malig-
nant well-differentiated serous tumors, whereas staining was stronger and present throughout the sample in some moderately differentiated serous carcinomas and in clear-cell carcinoma (representative samples shown in Fig. 6). On the tissue array, immunohistochemical analysis for p53 accumulation (i.e., mutation) was also performed. No p53 accumulation was observed in these tumors except for the moderately differentiated papillary serous cystadenocarcinoma (Fig. 6), in which <30% of the cells showed faint staining for p53, incompatible with a protein mutation. Among poorly differentiated or undifferentiated carcinomas, c-FLIP<sub>L</sub> staining was localized to the area of residual differentiation (representative sample shown in Fig. 6). Interestingly, poorly differentiated tumors showing c-FLIP<sub>L</sub> expression revealed no accumulation of p53, whereas those with no detectable c-FLIP<sub>L</sub> expression evidenced strong accumulation of p53 (representative samples shown in Fig. 6). Analysis of c-FLIP<sub>L</sub> and p53 expression in epithelial malignant tumors of the tissue array revealed a statistically significant inverse correlation (Table 2). Furthermore, intense staining was also ob-

Fig. 6 Immunohistochemical analysis of cellular FLICE-inhibitory protein long form (c-FLIP<sub>L</sub>) expression and p53 accumulation in ovarian epithelial tumors. Tissue array slides with fixed sections from 55 ovarian tumor specimens were stained with control antibody (A), goat anti-c-FLIP<sub>L</sub> antibody (B and C), or murine anti-p53 antibody (D). Shown are ×100 (A and B) and ×400 (C and D) original magnifications. C, higher magnification of boxed areas in B. Areas shown in D, stained with anti-p53 monoclonal antibody, are comparable to those shown in C stained with goat anti-c-FLIP<sub>L</sub> antibody.
Table 2  c-FLIP<sub>L</sub> expression versus p53 accumulation in epithelial ovarian carcinomas present in tissue array

<table>
<thead>
<tr>
<th>c-FLIP&lt;sub&gt;L&lt;/sub&gt;</th>
<th>No. of cases</th>
<th>Immunohistochemical staining for p53*</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>10</td>
<td>Negative 8 Nuclear accumulation 2</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>16</td>
<td>6 10</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>14 12</td>
<td>0.034</td>
</tr>
</tbody>
</table>

*Immunohistochemistry on paraffin-embedded tissue array sections using mouse monoclonal antibody DO7 (Novocastra) for p53 and goat polyclonal antibody C-19 to c-FLIP<sub>L</sub> (Santa Cruz Biotechnology).
†P value in χ<sup>2</sup> test.
Abbreviations: c-FLIP<sub>L</sub>, cellular FLICE-inhibitory protein long form.

served in germ-cell tumors with a lymphocytic component (i.e., dysgerminomas), and the staining was even stronger in samples with high lymphocyte infiltration. As expected, no p53 accumulation was detected in these tumors (Fig. 7).

**c-FLIP<sub>L</sub> Expression and p53 Accumulation Are Inversely Correlated.** To further confirm that c-FLIP<sub>L</sub> is preferentially expressed in ovarian carcinoma cells with wild-type p53 and that the p53 accumulation detected immunohistochemically in tissue array sections corresponds to a mutational state of p53, we analyzed immunohistochemically 27 cases of epithelial ovarian carcinoma (12 with wild-type p53 and 15 with mutated p53 status) for c-FLIP<sub>L</sub> and p53 expression. As expected, p53 accumulated at the nuclear level in all samples with the mutated protein, and again the inverse relationship between p53 mutation and c-FLIP<sub>L</sub> expression was statistically significant (Table 3).

The relationship among p53 status, p53 accumulation, and c-FLIP<sub>L</sub> expression was detailed in Table 4 for samples from six patients (three with wild-type p53 and three with mutated p53) for which the relative expression of the two molecules was also analyzed by Western blotting. Western blotting analysis of cellular lysates revealed overexpression of c-FLIP<sub>L</sub> in patients with wild-type p53, whereas, as expected, the p53 molecule was barely detectable (Fig. 8A). By contrast, Western blotting analysis of tissue lysates from patients with mutated p53 revealed overexpression of p53, compatible with an accumulation of the protein and with a low level of c-FLIP<sub>L</sub> (Fig. 8A), supporting the hypothesis that tumor cells with mutated p53 do not require the c-FLIP<sub>L</sub> mechanism to ensure resistance to apoptosis. Immunohistochemistry of archival material from the same six patients confirmed the inverse relationship between p53 and c-FLIP<sub>L</sub> expression in ovarian carcinoma cells (Fig. 8B). Note that the tumor from patient 2 contained a high percentage of stromal tissue, consistent with undetectable p53 levels in Western blotting (Fig. 8A). Nevertheless, c-FLIP<sub>L</sub> was detectable in both analyses, indicating that its expression was still relevant despite the relative proportion of tumor cells versus stromal tissue.

Because p53 accumulation observed in immunohistochemistry and in Western blotting corresponded in most cases with a mutated p53 state, we considered the data obtained with the tissue array together with the data from samples with known p53 status. Of 22 samples staining positive for c-FLIP<sub>L</sub>, 16 were negative for p53 staining (assuming a wild-type p53 status for all samples), whereas 6 were strongly positive for p53 staining (assuming that a mutated state corresponds to protein accumulation). By contrast, among the 31 samples staining negative for c-FLIP<sub>L</sub>, 10 were negative for p53 staining, whereas 21 showed p53 protein accumulation (i.e., mutation). This inverse relationship was highly significant (P = 0.0037).

Table 3  Immunohistochemical staining for c-FLIP<sub>L</sub> and p53 in paraffin-embedded sections from ovarian tumors with known p53 status

<table>
<thead>
<tr>
<th>c-FLIP&lt;sub&gt;L&lt;/sub&gt;</th>
<th>p53-negative cases (wild-type sequence)*</th>
<th>p53 nuclear accumulation (mutated sequence)*</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>12</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Negative</td>
<td>15</td>
<td>4 11</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>12 15</td>
<td>0.037</td>
</tr>
</tbody>
</table>

*All samples were analyzed for p53 nucleotide sequence; mutations were found in the most frequently affected exons (exons 5–8) of the gene.
†P value in χ<sup>2</sup> test.
Abbreviations: c-FLIP<sub>L</sub>, cellular FLICE-inhibitory protein long form.

**Fig. 7**  Immunohistochemical analysis of cellular FLICE-inhibitory protein long form and p53 expression in dysgerminomas. Tissue array slides were stained with hematoxylin and eosin to show tumor cellularity (A); control antibody (B), goat anti-c-FLIP<sub>L</sub> antibody (C), or murine anti p53 monoclonal antibody (D). Two representative dysgerminomas with rich (upper) or poor (lower) lymphocyte infiltration are shown. Original magnification, ×400.
DISCUSSION

In normal cells, when both procaspase-8 and c-FLIP\(_L\) are recruited to the DISC, they are cleaved and their cleavage intermediates remain bound to the receptor, preventing replacement by procaspase-8 and caspase-8 activation, thus inducing resistance to CD95-mediated apoptosis (20, 21). This mechanism of resistance has been described in lymphocytes at different stages of activation (20, 35). The same mechanism was observed in primary human B cells, where the transient protection afforded by c-FLIP may provide a safeguard mechanism against inappropriate cell death (36), and in neuroblastomas (37). In this study, we showed that ovarian cancer cells with wild-type p53 use this mechanism to block receptor-mediated apoptosis.

**Table 4** Clinicopathological features, p53 status, p53 protein accumulation, and c-FLIP\(_L\) expression of six patients with ovarian carcinoma

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Histological type and grade</th>
<th>p53 status</th>
<th>p53 accumulation</th>
<th>c-FLIP(_L) expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Serous, G2</td>
<td>Wild type</td>
<td>WB*</td>
<td>IHC†</td>
</tr>
<tr>
<td>2</td>
<td>Serous, G1</td>
<td>Wild type</td>
<td>WB*</td>
<td>IHC†</td>
</tr>
<tr>
<td>3</td>
<td>Serous, G3</td>
<td>Wild type</td>
<td>WB*</td>
<td>IHC†</td>
</tr>
<tr>
<td>4</td>
<td>Serous, G3</td>
<td>Exon 6, codon 195: ATC-ACC/Ile-Thr; exon 6, codon 213: CGA-CGG/polymorphism Arg-Arg</td>
<td>WB*</td>
<td>IHC†</td>
</tr>
<tr>
<td>5</td>
<td>Serous, G2</td>
<td>Exon 7, codon 235: AAC-CAC/Asn-His; Exon 7, codon 236: TAC-CAC/Tyr-His</td>
<td>WB*</td>
<td>IHC†</td>
</tr>
<tr>
<td>6</td>
<td>Serous, G3</td>
<td>Exon 5, codon 134: TTT-TCT/Phe-Ser; Exon 6, codon 213: CGA-CGG/polymorphism Arg-Arg</td>
<td>WB*</td>
<td>IHC†</td>
</tr>
</tbody>
</table>

* Western blotting of total cellular lysates using mouse monoclonal antibody DO7 (Novocastra) for p53 and rabbit polyclonal antibody H-202 to FLIP\(_L\) (Santa Cruz Biotechnology). For c-FLIP\(_L\) detection, see legend for Fig. 8A.
† Immunohistochemistry on paraffin-embedded sections using mouse monoclonal antibody DO7 (Novocastra) for p53 and goat polyclonal antibody C-19 to c-FLIP\(_L\).‡ Low reactivity in Western blotting due to low ratio of tumor cells vs. stromal tissue (see legend for Fig. 8B).

Abbreviations: c-FLIP\(_L\), cellular FLICE-inhibitory protein long form; ID, identification; WB, Western blotting; IHC, immunohistochemical staining.

**Fig. 8** Cellular FLICE-inhibitory protein long form (c-FLIP\(_L\)) expression correlates inversely with p53 mutation. A, Western blot analysis of proteins obtained from cryostatic sections from six ovarian carcinoma patients (see Table 2). Solubilized proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Blots were probed with rabbit anti-c-FLIP\(_L\) antibody, mouse anti-p53 monoclonal antibody, and as a control for protein loading, rabbit anti-β-actin antibody. B, immunohistochemical analysis of c-FLIP\(_L\) expression and p53 accumulation in archival samples from the six ovarian cancer patients. Formalin-fixed, paraffin-embedded tissue sections where stained with goat anti-c-FLIP\(_L\) antibody or murine anti-p53 monoclonal antibody. Original magnification, ×200.
In all of the cells tested in our study, DISC formation and caspase-8 activation after receptor triggering were inefficient. In OAW42 cells, c-FLIP_L was recruited to the DISC more efficiently than caspase-8, thus blocking the signal. Consistent with data obtained in other cellular systems (38–40), the block at the receptor level can be overcome by the use of protein synthesis inhibitors such as CHX. Furthermore, treatment with CHX before cross-linking and immunoprecipitation with anti-CD95 antibody dramatically decreases or eliminates c-FLIP_L recruitment to the DISC. Use of antisense oligonucleotides confirmed that c-FLIP_L is involved in OAW42 cells apoptosis resistance. Whereas antisense oligonucleotides down-modulated c-FLIP_L in both OAW42 and OVCAR3 cells, the down-modulation led to increased CD95-mediated apoptosis only in the wild-type p53 OAW42 cells and not in the mutated p53 OVCAR3 cells.

Immunohistochemical analysis of an ovarian tumor tissue array revealed positive staining for c-FLIP_L in nearly 50% of malignant epithelial ovarian tumors. The detection of the molecule in benign tumors (endometroid adenoacanthofibroma and Brenner tumors) and in borderline tumors suggests that c-FLIP_L might be up-modulated early during tumor progression. Among the epithelial malignant tumors, c-FLIP_L immunodecoration was present in well-differentiated carcinomas, with maximum expression in moderately differentiated carcinoma and in clear-cell carcinomas. The hypothesis that c-FLIP_L involvement in apoptosis inhibition is an early event in tumor progression is consistent with an observed decrease or even disappearance of c-FLIP_L expression in poorly differentiated or undifferentiated tumors. Concomitant with the disappearance of c-FLIP_L, p53 accumulation was observed, consistent with a mutation of the protein (41, 42). This inverse relationship was clearly demonstrated in the 27 cases of epithelial ovarian carcinoma in which a documented mutation of p53 corresponded to accumulation of the protein in 15 cases, and in accord with the data obtained in the tissue array, expression of c-FLIP_L decreased or totally disappeared as a function of p53 mutation. This inverse relationship, which was significant both in the tissue array samples (P = 0.034) and in the additional 27 cases analyzed (P = 0.037), implicates the CD95 block at the receptor level as a mechanism of apoptosis resistance in tumor cells in which a mutation of p53 has not yet occurred.

Among nonepithelial ovarian tumors, c-FLIP was expressed in 100% of dysergiminomas. These tumors are characterized by a stroma consisting of fibrous bands that almost invariably contain mature T lymphocytes (43). Staining for c-FLIP_L was more intense in the tumors with extensive lymphocyte infiltration, supporting the hypothesis that c-FLIP_L is up-modulated when tumor cells need protection from possible apoptotic stimuli in the context of a functional p53.

In addition to the direct involvement of c-FLIP_L in apoptosis impairment, the inefficient DISC formation observed in ovarian cancer cells raises the possibility that these cells are type II, characterized by only very low levels of caspase-8 activation at the DISC. Although this low caspase activity might fail to initiate a caspase cascade, it might nevertheless suffice to activate the mitochondria through BID. It has been suggested that mitochondria in type II cells may serve as a signal amplifier for low caspase activity generated at the DISC (16, 44). After CD95 cross-linking, we observed a decrease in the intact form of BID in all ovarian cells analyzed (data not shown). BID disappeared completely in OAW42 cells cotreated with anti-CD95 agonistic antibody and CHX, suggesting a total conversion to tBID. In these experimental conditions (anti-CD95 agonistic antibody plus CHX), total release of cytochrome c into the cytosol and subsequent total activation of caspase-3 was observed, supporting full mitochondrial activation and confirming the block upstream of mitochondria. Full activation of the mitochondrial pathway is consistent with the presence of a wild-type p53, which induces apoptosis by target gene regulation and transcription-dependent signaling. It has been reported that functionally active p53 may up-regulate c-FLIP expression in colon cancer, although the up-modulation in that model had no significant impact on FasL-induced apoptosis (45). This apparent contradiction rests in the simultaneous up-modulation of caspase-8 in these colon cancer cells, thus avoiding the stoichiometric inhibition due to c-FLIP. The up-modulation of c-FLIP_L only, without changes in caspase-8 levels, was observed in wild-type p53 ovarian cancer cells and is probably sufficient to inhibit caspase-8 recruitment to the DISC. Furthermore, it has recently been shown that a fraction of induced p53 translocates to the mitochondria of apotosing tumor cells, directly inducing permeabilization of the outer mitochondrial membrane by forming complexes with the protective Bcl-X_L or with Bcl-2 proteins, leading to a release of cytochrome c. Thus, mutations might represent “double-hits” by abrogating the transcriptional and mitochondrial apoptotic activity of p53 (46). This abrogation or alteration of p53 transcriptional activity in the case of protein mutation might also account for the down-modulation of c-FLIP_L that we observed in the majority of ovarian carcinomas bearing mutated p53. Further studies are needed to confirm this hypothesis.

The block at CD95 receptor level does not account for the resistance in all cell lines tested, but only for those bearing a wild-type p53. Indeed, malignant transformation may cause a decrease in CD95 expression, as observed in ovarian carcinoma cells obtained from ascitic fluids (47), but decreased CD95 expression does not always correlate with resistance. In fact, CD95 receptor expression is necessary but not sufficient for cell sensitivity (11, 47). Most likely, a different mechanism of resistance is at play in cells such as OVCAR3 in which a mutated p53 is present, and our preliminary data suggest that impairment of the mitochondrial pathway is involved.

Together, these data suggest the relevance of c-FLIP_L expression in protecting wild-type p53 human ovarian cancer cells from CD95-mediated apoptosis. Further studies are needed to evaluate the relevance of this block for the impaired apoptotic response to chemotherapy. Because down-modulation of the inhibitory molecule c-FLIP_L greatly enhances CD95-mediated apoptosis in cells in which p53 mutation has not yet occurred, new therapeutic strategies targeting c-FLIP_L can be envisaged.

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6 Our unpublished observations.

7 E. Balladore, manuscript in preparation, title has not been defined.
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CD95-Mediated Apoptosis Is Impaired at Receptor Level by Cellular FLICE-Inhibitory Protein (Long Form) in Wild-Type p53 Human Ovarian Carcinoma

Delia Mezzanzanica, Emanuela Balladore, Fabio Turatti, et al.


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