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Dysfunctional Apoptosome Activation in Ovarian Cancer: Implications for Chemoresistance[1]

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ABSTRACT

Alterations in the regulation of apoptosis may contribute to the pathogenesis of cancer and resistance of tumor cells to chemotherapy. In mammalian cells, nonreceptor-mediated apoptosis occurs predominantly via assembly of a cytochrome *c*-dependent apoptosome complex containing caspase-9 and apoptotic protease-activating factor-1 (Apaf-1). We show here that cytosolic extracts from human ovarian carcinoma cell lines and primary ovarian tumor samples are deficient in their ability to activate procaspase-9 in the presence of cytochrome *c* and dATP when compared with control extracts. SKOV3, a human ovarian carcinoma cell line with diminished apoptosome activity, was significantly more resistant to chemotherapy-induced apoptosis than cell lines with functional Apaf-1 activity. This dysfunctional apoptosome activity was not explained by reduced expression levels of caspase-9 or Apaf-1. Moreover, expression levels of known inhibitors of the apoptosome, including heat shock protein 70, heat shock protein 90, or X-linked inhibitor of apoptosis, did not correlate with functional activity of the apoptosome. SKOV3, an ovarian cancer cell line with dysfunctional apoptosome activity, retains the ability to form the Apaf-1 oligomer; however, there is a diminished amount of caspase-9 in the apoptosome. The reduction in the amount of caspase-9 in the apoptosome in the SKOV3 cell line was associated with diminished caspase-3 activity. Dysfunctional apoptosome activation may contribute both to the pathogenesis of ovarian carcinoma and to chemoresistance.

INTRODUCTION

Epithelial ovarian cancer is the leading cause of death of women with gynecological cancers and is the fourth leading cause of cancer death among women in the United States. Primary cytoreductive surgery followed by platinum based chemotherapy (*e.g.*, cisplatin, carboplatin) is the standard of care for treatment of ovarian cancer. This treatment is associated with high response rates, but the disease is characterized by recurrence and the subsequent development of resistance to chemotherapy. Only a minority of patients survive 5 years. Resistance to chemotherapy has been associated with decreased susceptibility to apoptosis, introducing the possibility that cell death determinants may influence the outcome of treatment (1).

The family of mammalian caspases (aspartate-specific cysteine proteinases) represents the effector arm of the apoptotic program. Caspases are expressed in cells as inactive precursors, which are processed following stimulation with apoptotic signals into mature forms composed of a tetramer of two large and two small subunits (2). Apoptosis occurs via a proteolytic cascade in which upstream initiator caspases (caspase-8, -9, and -10) activate downstream effector caspases (caspase-3, -6, and -7; Refs. 2–4). *In vivo*, this results in the cleavage of target proteins and the demise of the cell. The initiator caspases are activated through intrinsic autocatalytic activity in conjunction with other proteins with which they form complexes known as apoptosomes (5).

Two distinct pathways leading to apoptosis have been delineated. Both converge on the activation of downstream caspases. The extrinsic pathway involves ligation of death receptors such as Fas or tumor necrosis factor receptor-1 by their ligands, resulting in the recruitment of procaspase-8 or procaspase-10 to the receptor complex through the adaptor Fas-associated death domain (2). Following oligomerization, procaspase-8 or procaspase-10 are self-activated presumably through induced proximity of procaspase molecules (6). These active species then cleave their downstream targets, which include effector caspases. A second pathway independent of death receptors leads first to mitochondrial release of cytochrome *c*. In the presence of dATP/ATP, cytochrome *c* binds to Apaf-1,[3] which then oligomerizes and binds procaspase-9, resulting in the formation of an apoptosome containing Apaf-1 and caspase-9 (5, 7). Biochemical studies have shown that Apaf-1 oligomerizes to a M_r 700,000-1,400,00 complex that contains both procaspase-9 and processed caspase-9 (7–9). There is some controversy over whether the apoptosome also contains fully processed caspase-3 and caspase-7 (7, 9). Apaf-1 oligomerization allows proximity and enzymatic self-activation of bound caspase-9. There is controversy over whether the enzymatic activity of caspase-9 is attributed to cleaved caspase-9 bound to the apoptosome (8) or if processed caspase-9 that has been released from the apoptosome is also enzymatically active (10).

Evaluation of the function of the apoptosome complex in tumor specimens has been limited. Recently, investigators have shown that Apaf-1 is inactivated in malignant melanoma and that its inactivity may contribute toward the aggressive, chemoresistant phenotype associated with this disease (11). In the present study, we assess the role of the Apaf-1/caspase-9 apoptosome in epithelial ovarian cancer cell lines as well as in primary ovarian tumors.

MATERIALS AND METHODS

Cell Lines and Immunoblotting. Ovarian cancer cell lines A2780, CaOV3, MDAH 2774, and SKOV3, and Jurkat cells were obtained from the American Type Culture Collection. Heat shock of Jurkat cells was performed as described (12). Cytosolic extracts were prepared as described (12). Immunoblotting analysis was performed with relevant antibodies. Apaf-1 was detected with a monoclonal antibody obtained from Yuri Lazebnik (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Monoclonal antibodies for caspase-9, Hsp70, and Hsp90 were obtained from Stressgen (Victoria, Canada). The monoclonal antibody for XIAP was obtained from BD Transduction (Franklin Lakes, NJ). The monoclonal antibody for β-tubulin was obtained from Sigma Chemical Co.-Aldrich (St. Louis, MO). Western blots were developed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). Densitometry analysis was performed using Scion Image Beta 4.02 software from Scion Corp (Frederick, MD).

***In Vitro* Chemotherapy-induced Cell Death and Caspase Activation Assays.** Ovarian cancer cells were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100

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[3] The abbreviations used are: Apaf-1, apoptotic protease-activating factor-1; Hsp70, heat shock protein 70; Hsp90, heat shock protein 90; WDR, WD-40 repeat region; XIAP, X-linked inhibitor of apoptosis.

mg/ml; Life Technologies, Inc., Carlsbad, CA). Cells were plated, and 24 h after plating, 10 μg/ml cisplatin (Bristol-Myers Squibb, Princeton, NJ) was added to the culture medium. The percentage of apoptotic cells was determined at the indicated time points by nuclear propidium iodide staining as described previously (14). In this assay, apoptotic cells exhibit a sub-Go DNA content resulting from DNA fragmentation (15). For caspase activation assays, cells were harvested with trypsin and then double stained with propidium iodide and a carboxyfluorescein-labeled caspase substrate for caspase-9 (FAM-LEHD-FMK; Intergen, Purchase, NY). Processing of this substrate was measured by flow cytometry. Analysis was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Patient Sample Preparation. Patient samples were obtained from patients with primary or recurrent epithelial ovarian cancer admitted to the University of Michigan Medical Center. In conjunction with institutional review board approval, samples were obtained at the time of primary surgery or at the time of paracentesis for diagnosis of recurrent disease. For solid tumor samples, the tumor tissue was minced and incubated with collagenase type 3 (Worthington Biochemical Corp, Lakewood, NJ) at 37°C. Cellular material was pelleted, washed with M199 medium, RBCs were lysed by hypotonic lysis, and cell pellet aliquots were frozen in liquid nitrogen. For ascites samples, cells were pelleted and processed as described above. Primary ovarian surface epithelial cells were collected from normal ovaries using the scrape technique as described (16). Cytosolic lysates were prepared as described (17).

***In Vitro* Caspase-9 and Caspase-3 Assay.** Cytosolic extracts were prepared from cell lines and primary tumor cultures as described (17). The *in vitro* caspase-9 and caspase-3 assays were performed as described previously (17, 18). Reactions were stopped with 5 × SDS loading buffer [250 mM Tris-Cl (pH 6.8), 500 mM DTT, 10% SDS, 0.5% bromophenol blue, and 50% glycerol], boiled, and loaded onto a 12% polyacrylamide/SDS gel. Gels were dried and exposed for autoradiography.

Reverse Transcription-PCR Analysis of Cell Lines and Human Samples. RNA was extracted from human embryonic kidney 293T, A2780, MDAH 2774, CaOV3, and SKOV3 cell lines, and from human ovarian cancer samples using a commercially available kit (Qiagen, Valencia, CA). cDNA was prepared using these samples as templates according to protocols provided with a commercially available kit (Promega, Madison, WI). Apaf-1 cDNAs were detected using the specific primers: C1, 5′-CAGCTGATGGAACCTTA-AAGC-3′and C2, 5′-GTCTGGTCATCAGAAGATGTC-3′, and PCR conditions as described (18).

Fractionation of Cytosolic Extracts by Gel Filtration. S-100 cytosolic extracts were prepared as described (17) and incubated at 30°C for 60 min in the presence or absence of 10 μg/ml cytochrome *c* and 1 mM dATP. Lysate (2 mg) was loaded on a Superdex-200 HR gel filtration column (Amersham Pharmacia Biotech) pre-equilibrated with Buffer A [20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride] at a flow rate of 0.5 ml/min using a Bio-Rad BioLogic HR Workstation. The column was calibrated with an Amersham Pharmacia Biotech HMW gel filtration protein standards kit (thyroglobulin M_r = 669,000, ferritin M_r = 440,000, catalase M_r = 232,000, and BSA M_r = 66,000). After discarding the majority of the void volume, fractions of 400 μl were collected. Aliquots of 50 μl from each fraction were resolved by SDS-PAGE followed by immunoblotting with monoclonal antibodies for Apaf-1, caspase-9, XIAP, Hsp70, and Hsp90.

RESULTS

Differential Response to Cisplatin-induced Apoptosis and Caspase Activation in Ovarian Cell Lines. We tested the sensitivity of A2780, MDAH 2774, CaOV3, and SKOV3 ovarian cell lines to cisplatin-induced apoptosis. After incubation with the drug (10 μg/ml), the apoptotic fraction was determined by flow cytometry at 24 and 48 h after treatment. By 48 h, cisplatin induced apoptosis in 50–70% of cells in the A2780, MDAH 2774, and CaOV3 cell lines (Fig. 1*A*). In contrast, almost 90% of the SKOV3 cells were resistant to cisplatin-induced apoptosis after 48 h of treatment (Fig. 1*A*). To determine whether chemotherapy-induced apoptosis correlates with caspase activation, cells were analyzed for caspase-9 activity. By 24 h, 12–56% of A2780, 2774, and CaOV3 cells demonstrated caspase-9 activity, which increased to 35–56% after 48 h of treatment (Fig. 1*B*). However, in the chemoresistant SKOV3 cell line, <3% of the cells showed detectable caspase-9 activity even after 48 h of treatment (Fig. 1*B*). To directly assess caspase-9 activation, cytosolic extracts from cell lines were immunoblotted for caspase-9 after treatment with cisplatin. A2780, MDAH 2774, and CaOV3 cells all demonstrated cleavage of endogenous caspase-9 indicated by the presence of the intermediate p35 proteolytic fragment (Fig. 1*C*). In contrast, treatment

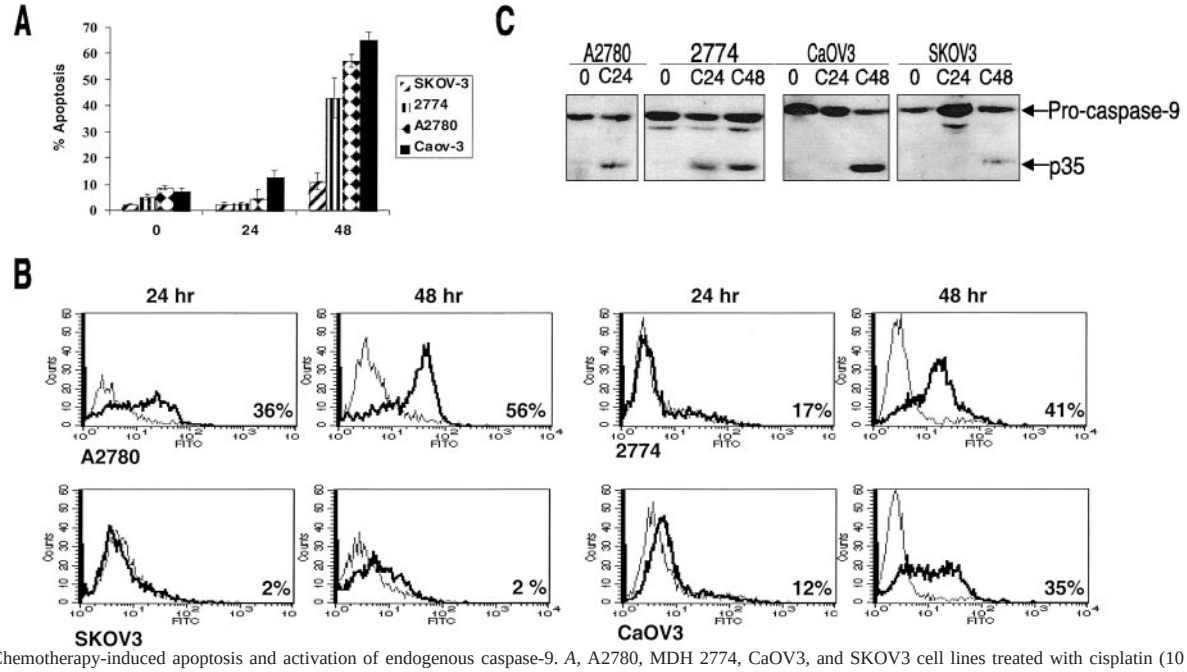

Fig. 1. Chemotherapy-induced apoptosis and activation of endogenous caspase-9. *A*, A2780, MDH 2774, CaOV3, and SKOV3 cell lines treated with cisplatin (10 μg/ml) were analyzed for apoptosis by flow cytometry after 24 and 48 h. Viability represents the percentage of nonapoptotic cells. Values represent the mean from three separate experiments; *bars*, ± SD. *B*, A2780, MDH 2774, CaOV3, and SKOV3 ovarian carcinoma cell lines were treated with cisplatin (10 μg/ml) and analyzed for *in vivo* caspase-9 activity with the cell permeable fluorogenic substrate FAM-LEHD-FMK. *C*, after treatment with cisplatin (10 μg/ml), cytosolic lysates were prepared at the indicated times. Immunoblot analysis for caspase-9 was performed with a monoclonal antibody. In A2780 cells, cell death by 48 h exceeded 90%, precluding caspase-9 analysis at this time point.

of SKOV3 cells with cisplatin resulted in minimal processing of endogenous caspase-9 (Fig. 1*C*).

The Inability to Undergo Cisplatin-induced Caspase-9 Activation Correlates with Deficient Apaf-1 Activity in Ovarian Cell Lines. Apaf-1 is a cytosolic factor that activates caspase-9 (4). We next tested whether the differential response of ovarian cell lines to cisplatin-induced caspase-9 activation correlated with the ability to process procaspase-9 *in vitro* as a surrogate for Apaf-1 activity. Cytosolic extracts were incubated with *in vitro* translated ^{35}S procaspase-9, and caspase cleavage was determined after addition of cytochrome *c* and dATP. As shown in Fig. 2*A*, cytosolic extracts from 293T, A2780, 2774, and CaOV3 cells induced the activation of procaspase-9 in a cytochrome *c* and dATP dependent fashion as indicated by the appearance of the intermediate p35 proteolytic fragment. In contrast, extracts from SKOV3 did not activate procaspase-9 (Fig. 2*A*).

To assess the function of endogenous caspase-9 in the cell lines, we next tested the ability of the cytosolic lysates to process *in vitro* translated ^{35}S procaspase-3. As shown in Fig. 2*B*, cytosolic extracts from 293T, A2780, 2774, and CaOV3 cells induced the activation of procaspase-3 in a cytochrome *c*- and dATP-dependent fashion. Extracts from SKOV3 did not activate procaspase-3.

We performed immunoblotting to determine the levels of Apaf-1 in the ovarian cell lines. Apaf-1 was detected in all of the cell lines tested (Fig. 2*C*). Expression levels of Apaf-1 varied slightly but did not correlate with the ability of the cytosolic extracts to activate procaspase-9, procaspase-3, or with the pattern of chemosensitivity.

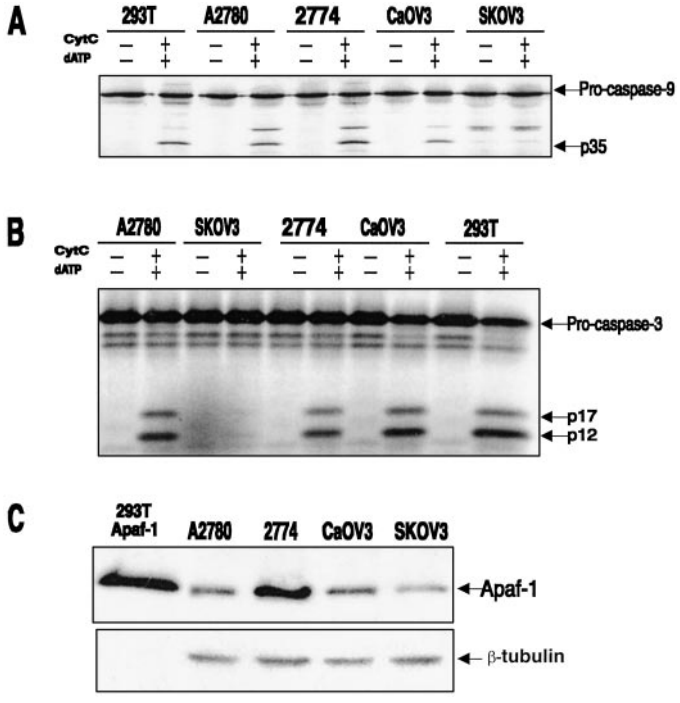

Fig. 2. Expression of Apaf-1 and activation of procaspase-9 in ovarian carcinoma cell lines. *A*, S100 cytosolic extracts (40 μg protein) prepared from ovarian cancer cell lines and control 293T cells were incubated with *in vitro* translated ^{35}S procaspase-9, with or without 8 μg/ml cytochrome *c* and 1 mM dATP at 30° for 30 min. Caspase-9 was resolved by SDS-PAGE. *B*, S100 cytosolic extracts (40 μg protein) prepared from ovarian cancer cell lines and control 293T cells were incubated with *in vitro* translated ^{35}S procaspase-3, with or without 8 μg/ml cytochrome *c* and 1 mM dATP at 30° for 30 min. Caspase-3 was resolved by SDS-PAGE. *C*, S100 cytosolic extracts (40 μg protein/sample) from ovarian cancer cell lines were resolved by SDS-PAGE and analyzed by immunoblotting for endogenous Apaf-1. Extracts from control 293T cells transfected with Apaf-1XL identify correct bands. Immunoblotting for β-tubulin as a loading control was performed as well. β-Tubulin levels for the positive control were undetectable, because only 1 μg of protein was loaded.

Cell Extracts from Primary Ovarian Tumors Are Deficient in Apaf-1 Activity. We next examined the ability of cell extracts derived from primary ovarian tumors to activate procaspase-9 using the *in vitro* assay. Twenty-eight ovarian carcinoma samples were harvested and cytosolic extracts were prepared. Thirteen samples were derived from patients with primary ovarian cancer before treatment with chemotherapy, and 15 samples were harvested from patients with recurrent ovarian cancer after treatment with chemotherapy. Sixteen samples were obtained as ascites, and 12 samples were obtained as solid tumor biopsies. Twenty-one of 28 samples analyzed (77%) were deficient in Apaf-1 activity in that they demonstrated no detectable ability to activate procaspase-9 in the presence of cytochrome *c* and dATP (Fig. 3*A*). Cytosolic extracts from control 293T cells and normal ovarian epithelial cells activated procaspase-9 (Fig. 3*A*). Five of 13 of the primary ovarian tumor samples and 2 of 15 of the recurrent ovarian tumor samples induced the activation of procaspase-9 in a cytochrome *c*- and dATP-dependent fashion.

Similarly, a subset of patient samples was analyzed for the ability to cleave *in vitro*-translated ^{35}S procaspase-3 in the presence of cytochrome *c* and dATP (Fig. 3*B*). Three patient samples with the ability to cleave radiolabeled procaspase-9 (Fig. 3*A*, *#1*, *4*, and *6*) and 4 patient samples that demonstrated no ability to cleave radiolabeled procaspase-9 (Fig. 3*A*, *#24*, *33*, *39*, and *40*) were selected. Two of the 3 samples that demonstrated procaspase-9 activation were not able to cleave procaspase-3. All of the samples that did not process procaspase-9 also did not cleave procaspase-3. Although a larger number of patient samples will need to be analyzed to make definitive conclusions about the clinical significance of deficient apoptosome activity in this disease, these results suggest that in the majority of primary and recurrent ovarian cancers, the apoptosome is nonfunctional.

Apaf-1 Levels Do Not Correlate with Ability to Activate Caspase-9 in Primary Ovarian Tumors. We next determined the levels of Apaf-1 protein in extracts of the primary ovarian tumors to determine whether differences in the amount of Apaf-1 present explains the results presented above. Immunoblotting analysis showed that Apaf-1 was expressed in all of the primary tumor samples (Fig. 4*A*). Although the amounts of Apaf-1 varied, protein levels did not correlate with cytosolic activation of procaspase-9 (Fig. 3).

Several isoforms of human Apaf-1 have been described based on differential splicing of the *Apaf-1* gene (19). Apaf-1XL contains an insertion of 43 amino acids creating an additional WD-40 repeat in the COOH terminus (Fig. 4*B*) required for functional activity (18). Therefore, we determined whether ovarian cell lines and primary tumors express sequences encoding this isoform. As seen in Fig. 4*C*, all of the tumor samples analyzed expressed both Apaf-1 isoforms (with and without the additional WD-40 repeat), although the transcript corresponding to the Apaf-XL isoform was most abundant. This pattern of Apaf-1 expression is similar to that observed in normal human tissues (18). Although differences in relative amounts of Apaf-1 isoforms were seen between tumor samples, these differences were not predictive of functional or defective Apaf-1 activity.

Expression of Heat Shock Proteins and XIAP Does Not Correlate with Functional Activity of the Apoptosome in Ovarian Cancer. Recently, several investigators have described negative regulation of the Apaf-1 apoptosome complex by Hsp70 and Hsp90 (12, 13). Hsp70 appeared to bind to Apaf-1 but not to procaspase-9, preventing recruitment of caspases to the apoptosome complex (12). Hsp90 appeared to inhibit cytochrome *c*-mediated oligomerization of Apaf-1 and thereby activation of procaspase-9 (13). XIAP has been shown to inhibit caspase-9 processing of caspase-3 by binding to and inhibiting processed mature caspase-9 (20). Immunoblot analysis of 293T, A2780, MDAH 2774, and CaOV3 cell lines revealed expres-

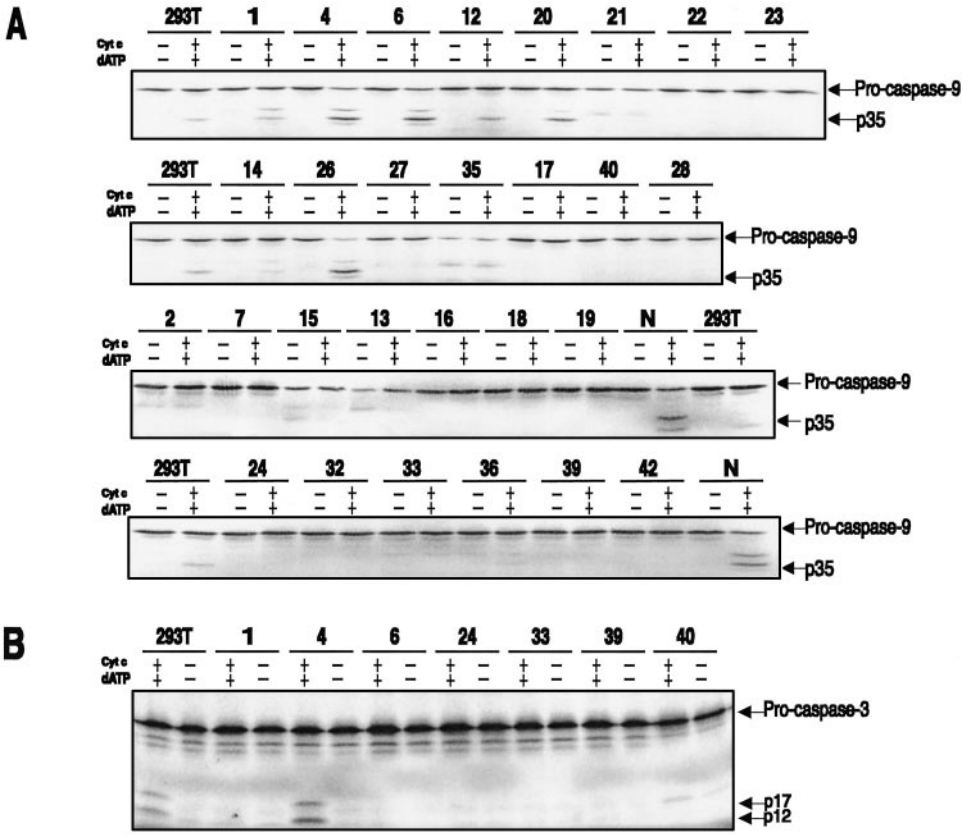

Fig. 3. Activation of procaspase-9 in human ovarian carcinoma. *A*, tumor samples from patients with primary or recurrent epithelial ovarian carcinoma were used to prepare S100 cytosolic extracts. Protein (40 μg) per sample was incubated with *in vitro* translated ^{35}S procaspase-9 in the presence or absence of 8 μg/ml cytochrome *c* and 1 mM dATP at 30° for 30 min. Caspase-9 was resolved by SDS-PAGE. Patient samples are coded by the numbers indicated at the top of each blot. *B*, S100 cytosolic extracts from selected patients were incubated with *in vitro* translated ^{35}S procaspase-3 in the presence or absence of 8 μg/ml cytochrome *c* and 1 mM dATP at 30° for 30 min. Caspase-3 was resolved by SDS-PAGE.

sion of Hsp70, Hsp90, and XIAP; however, expression levels of these proteins did not correlate with functional activity of Apaf-1 (Fig. 5, *A*, *B*, and *E*). Similarly, immunoblot analysis of primary ovarian carcinoma samples revealed expression of Hsp70, Hsp90, and XIAP in all of the samples, but again, expression levels did not correlate with functional activity of Apaf-1 (Fig. 5, *C*, *D*, and *F*).

SKOV3 Cells Retain the Ability to Form Apaf-1 Oligomers. To determine whether the nonfunctional apoptosome activity of SKOV3 cells (inability to cleave *in vitro* translated caspase-9) is explained by defective formation of the approximately M_r 700,000–1,400,00 Apaf-1 oligomer described previously (7–9), we used size exclusion chromatography to fractionate cytosolic extracts from two representative ovarian cancer cell lines. After activation with cytochrome *c* and dATP to induce Apaf-1 oligomerization or a similar treatment in the absence of cytochrome *c* and dATP as a control, fractions separated by gel filtration chromatography were analyzed for Apaf-1 expression by immunoblotting with anti-Apaf-1 antibody. In the absence of cytochrome *c* and dATP, both A2780 and SKOV3 cell lines demonstrated Apaf-1 elution in fractions corresponding to ~M_r 200,000 protein, which is consistent with the monomeric form of this protein (Fig. 6, *B* and *D*). Incubation of extracts with cytochrome *c* and dATP resulted in a shift in the Apaf-1 elution profile so that the majority of

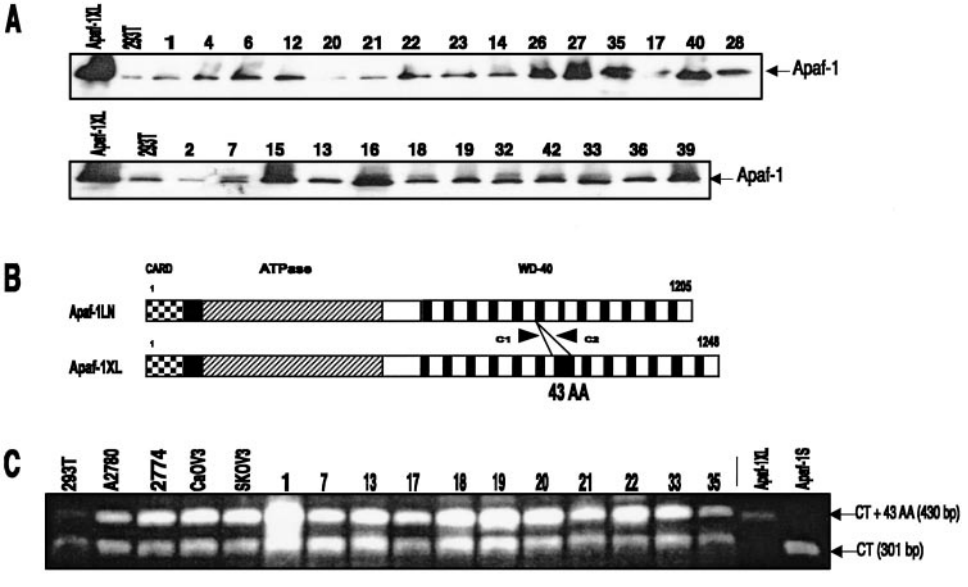

Fig. 4. Expression of Apaf-1. *A*, S100 cytosolic extracts prepared from human tumor samples (40 μg protein/sample) were analyzed by immunoblotting for Apaf-1 using a monoclonal antibody. *B*, schematic representation of Apaf-1 isoforms examined in this study. The caspase recruitment domain, ATPase domain, and WDRs are shown. The presence or absence of the 43-amino acid insert between the fifth and sixth WDRs. C1/C2 represent the primers used to amplify the regions flanking the COOH-terminal insert. *C*, reverse transcription-PCR analysis of the ovarian cancer cell lines and representative ovarian cancer tumor samples using primers C1 and C2. The last two *lanes* are positive controls, using cDNAs corresponding to Apaf-1XL and Apaf-1S (Apaf-1 isoforms with and without the extra WD-40 repeat) as templates.

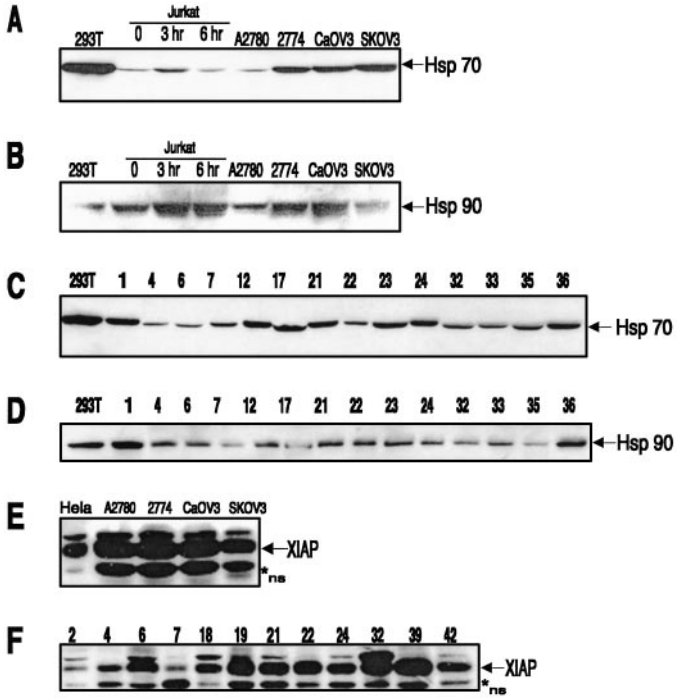

Fig. 5. Cytosolic inhibitors of the Apaf-1 apoptosome: heat shock proteins and XIAP. *A*, immunoblot for Hsp70 in the indicated cell lines. Jurkat cells expressing inducible Hsp70 after heat shock (45 min at 42°C) and recovery at 37°C for 3 and 6 h were used as a positive control. Extracts from ovarian cancer cell lines were analyzed without heat shock. *B*, immunoblot analysis for Hsp90 in the indicated cell lines. *C*, immunoblot analysis for Hsp70 in representative human ovarian carcinoma tumor samples. Only patient samples with adequate sample quantity were analyzed. *D*, immunoblot analysis for Hsp90 in representative human ovarian carcinoma samples. *E*, immunoblot analysis for XIAP in the indicated cell lines. *F*, immunoblot analysis for XIAP in representative human ovarian carcinoma samples.

Apaf-1 eluted in fractions 3–7, corresponding to approximately M_r 700,000–1,400,00 (Fig. 6, *A* and *C*). These results indicate that the nonfunctional apoptosome activation of SKOV3 is not attributable to the inability of Apaf-1 to oligomerize in response to cytochrome *c* and dATP. Preliminary experiments using extracts from representative patient samples show similarly that in samples with functional (#4) and nonfunctional (#17) apoptosome activation, Apaf-1 is able to oligomerize in response to cytochrome *c* and dATP (data not shown).

SKOV3 Cells Express a Diminished Amount of Caspase-9 in the Apoptosome. The apoptosome has been characterized as an Apaf-1-containing complex that recruits and activates procaspase-9, which then activates caspase-3 (19). To determine whether the Apaf-1 apoptosome contains activated caspase-9, immunoblot analysis was performed on the fractions containing the Apaf-1 oligomer for the cell lines A2780 and SKOV3. As seen in Fig. 7*A*, in the A2780 cell line after dATP activation, ~50% of the procaspase-9 was processed to its catalytically active large subunits (p35/p37), which eluted with the approximately M_r 700,000–1,400,000 apoptosome complex. Fractions 15–19 contain procaspase-9, and cleaved caspase-9 that has been released from the Apaf-1 apoptosome. In contrast, in the SKOV3 cell line, a negligible amount of procaspase-9 was processed to its catalytically active large subunits (p35/p37) and subsequently coeluted with the approximately M_r 700,000–1,400,000 apoptosome complex (Fig. 7*C*). Most of the caspase-9 remained in its proform and eluted in fractions 15–19. These results suggest that in the chemoresistant SKOV3 cell line, dysfunctional apoptosome activation is associated with a decrease in the amount of cleaved caspase-9 bound to the Apaf-1 apoptosome.

To determine whether cleaved caspase-9 released from the Apaf-1 apoposome remains enzymatically active and can subsequently cleave caspase-3, fractionated S100 cytosolic lysates from the cell lines A2780 and SKOV3 were incubated with *in vitro* translated ^{35}S-labeled caspase-3 in the presence of cytochrome *c*/dATP. In the

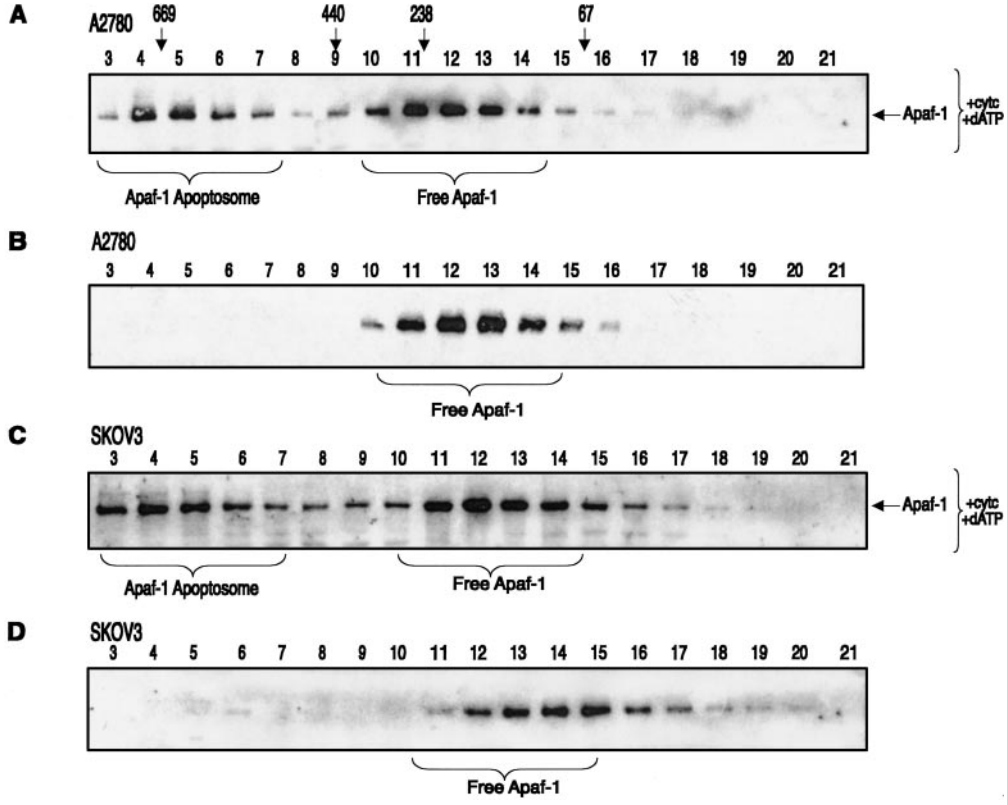

Fig. 6. Oligomerization of Apaf-1. A2780 S100 lysates (*A* and *B*) and SKOV3 S100 lysates (*C* and *D*) were incubated for 30 min in the presence or absence of cytochrome *c*/dATP then fractionated on a Superdex 200 HR column. Equal amounts (50 μl) of each fraction were resolved by SDS-PAGE, and Apaf-1 was detected by immunoblotting with a monoclonal antibody. The elution profiles and sizes of selected standards are indicated by *arrowheads on top of A*.

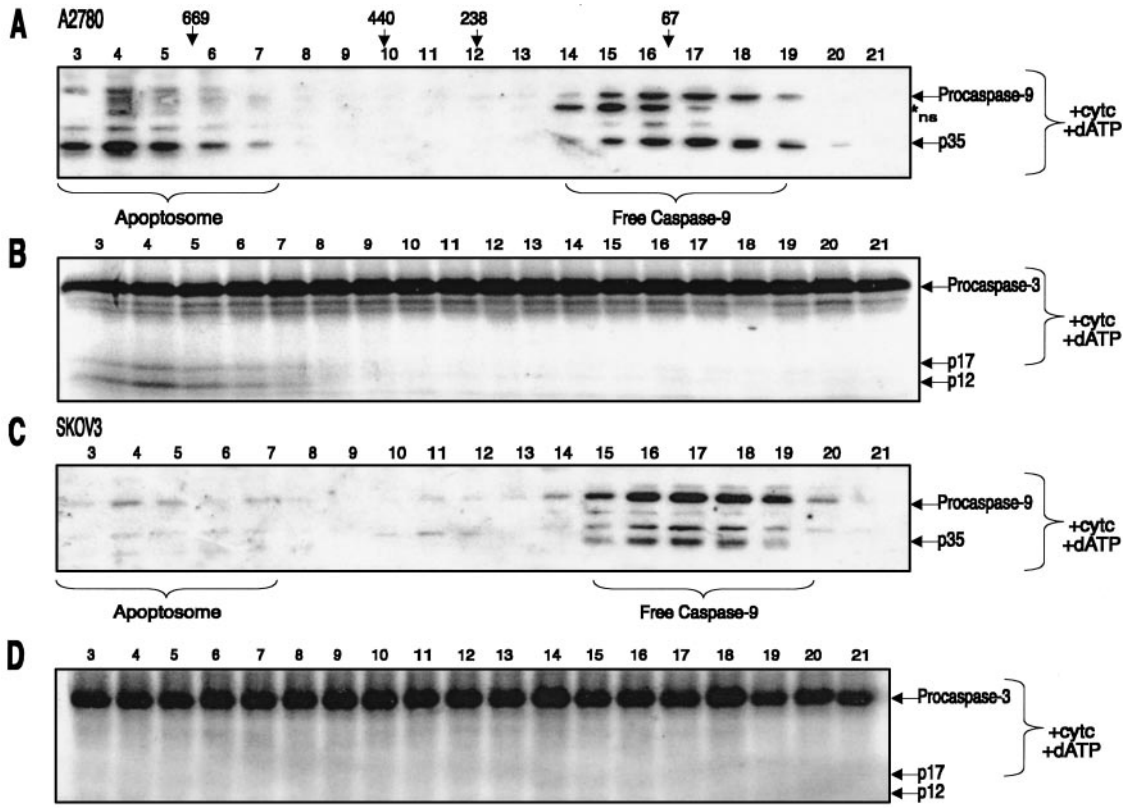

Fig. 7. Analysis of caspase-9 activity. A2780 S100 lysates (*A*) and SKOV3 S100 lysates (*C*) were incubated for 30 min in the presence or absence of cytochrome *c*/dATP then fractionated on a Superdex 200 HR column. Equal amounts (50 μl) of each fraction were resolved by SDS-PAGE, and caspase-9 was detected by immunoblotting with a monoclonal antibody. The elution profiles and sizes of selected standards are indicated by *arrowheads on top of A*. Fractionated S100 lysates from A2780 (*B*) and SKOV3 (*D*) were incubated with *in vitro* translated ^{35}S procaspase-3 in the presence or absence of 8 μg/ml cytochrome *c* and 1 mM dATP at 30° for 30 min. Caspase-3 was resolved by SDS-PAGE.

A2780 cell line, only fractions 3–7 containing the Apaf-1 oligomer as well as cleaved caspase-9 were able to cleave caspase-3. Fractions 15–19, containing monomeric caspase-9 along with cleaved caspase-9 that had been released from the Apaf-1 apoptosome, did not cleave caspase-3. In the SKOV3 cell line, none of the fractions were able to cleave caspase-3. These findings are consistent with previous work by investigators that showed that cleaved caspase-9 is active and can activate downstream caspase-3 only when it is bound to the Apaf-1 apoptosome (8).

As mentioned above, several investigators have reported negative regulation of the Apaf-1 apoptosome complex by Hsp70, Hsp90 (12, 13), and XIAP (20). To determine whether these proteins play a role in the function of the Apaf-1 apoptosome complex in ovarian cancer, cytosolic fractions from A2780 and SKOV3 activated with cytochrome *c* and dATP were separated by gel filtration chromatography and were analyzed for expression of Hsp70, Hsp90, and XIAP by immunoblotting. Expression of Hsp70 was equivalent between the A2780 and SKOV3 extracts across all of the fractions. Hsp90 and XIAP did not coelute with the Apaf-1 apoptosome (fractions 4–6) in either cell line, with the majority eluting in fractions 6–11. These results suggest that Hsp70, Hsp90, and XIAP do not interfere with the formation of the Apaf-1 oligomer in ovarian cancer (data not shown).

DISCUSSION

Alterations in the regulation of apoptosis may contribute both to the pathogenesis and the development of chemoresistance in ovarian cancer. Chemotherapy-induced apoptosis is thought to occur via assembly of a cytochrome *c*-dependent apoptosome complex containing the proteins Apaf-1 and caspase-9 (3). Several studies have indicated that cells lacking procaspase-9 or Apaf-1 exhibit resistance to chemotherapy (11, 21–23). However, evaluation of the expression of Apaf-1 and procaspases -2, -3, -7, -8, and -9 in leukemia patients did not correlate with clinical response to therapy (24). In this study, we show that the majority of primary ovarian tumors and a subset of ovarian cell lines are deficient in Apaf-1 activity. In tumor specimens, processing of procaspase-9 was only detected in 7 of 28 samples. Recognizing that processed caspase-9 may be enzymatically active only when retained in the apoptosome (8), we additionally analyzed a subset of those tumor specimens that cleaved procaspase-9 to determine whether they were capable of processing procaspase-3. Interestingly, 2 of 3 samples were unable to process procaspase-3. The absence of procaspase-3 activation suggested that although procaspase-9 was cleaved, it was not retained in the apoptosome complex.

In the ovarian cell lines, deficient Apaf-1 activity *in vitro* was linked to reduced caspase-9 activation *in vivo* and reduced susceptibility to cisplatin-induced apoptosis. The regulation of caspase-9 activity is complex, but it is thought to be primarily controlled by Apaf-1. Cytochrome *c* (released from damaged mitochondria) and dATP/ATP are critical factors that regulate the activity of Apaf-1 (4). Because our assay for Apaf-1 activity was performed in the presence of cytochrome *c* and dATP, our results suggest that deficient Apaf-1 activity in ovarian tumors is attributable to a defect in the apoptotic pathway downstream of cytochrome *c* release. However, it is possible that in ovarian tumor cells additional defects contribute to deregulation of the Apaf-/caspase-9 pathway. For example, certain ovarian tumors overexpress Bcl-x_L (25), which inhibits caspase-9 activation, by preventing the release of cytochrome *c* from mitochondria (26, 27).

One possible explanation for deficient Apaf-1 activity in ovarian tumors is the presence of altered forms of Apaf-1 or caspase-9. Other investigators have described the presence of an alternatively spliced short form of caspase-9 (termed caspase-9b) that acts as a dominant-negative fashion (28). However, immunoblotting analysis of the ovarian cancer cell lines tested did not reveal the presence of any short isoform of caspase-9 (Fig. 1*C*). Deletion or silencing of Apaf-1 has been reported in metastatic melanomas (11). However, in our work all of the tumors and cell lines evaluated expressed Apaf-1 protein. It is possible that ovarian tumors contain subtle genetic alterations such as point mutations that impair the activity of Apaf-1. Despite this possibility, other investigators have failed to find consistent Apaf-1 mutations in a variety of cancers including lymphoma, and pancreatic, germ cell, cervical, or colorectal carcinomas (29, 30). Alternative splicing can influence the ability of Apaf-1 to activate caspase-9 (18). Isoforms expressing 12 WD-40 COOH-terminal repeats demonstrate a decreased cytochrome *c*-/dATP-dependent caspase activation as compared with isoforms containing 13 WD-40 repeats (18). All of the ovarian cancer cell lines analyzed expressed RNA encoding Apaf-1 isoforms with both the 12 and 13 WD-40 COOH-terminal repeats, suggesting that the deficient Apaf-1 activity is not attributable to the absence of the functional isoform of Apaf-1.

Inhibitors of the apoptosome including Hsp70, Hsp90, and XIAP have been described recently (12, 13, 20). Deregulated expression of any of these inhibitors could explain the deficiency of Apaf-1 activity in ovarian tumors. Hsp70 has been shown to bind to Apaf-1 but not to procaspase-9, and it appears to prevent the recruitment of caspases to the apoptosome complex (12). Hsp90 has been shown to inhibit cytochrome *c*-mediated oligomerization of Apaf-1 and subsequent activation of procaspase-9 (13). XIAP has been shown to bind to and inhibit processed, mature caspase-9 (20). Expression levels of these proteins in ovarian cell lines and primary tumors did not correlate with functional activity of Apaf-1 (Fig. 5). Moreover, Hsp70 did not coelute with the Apaf-1 oligomer in either the A2780 or the SKOV3 cell line, and despite the expression of Hsp90 in both cell lines, the Apaf-1 oligomer still formed normally (data not shown; Fig. 6). Similarly, XIAP did not coelute with the Apaf-1 oligomer in either the A2780 or the SKOV3 cell line (data not shown).

In the chemoresistant SKOV3 cell line, which demonstrates deficient Apaf-1 activity, we show that the ability to form the Apaf-1 apoptosome is retained; however, there is a decrease in the amount of cleaved caspase-9 bound to the Apaf-1 apoptosome. There is some controversy concerning the enzymatic activity of processed caspase-9 that has been released from the apoptosome (8, 10). Our data indicates that only cleaved caspase-9 bound to the Apaf-1 apoptosome can activate downstream caspase-3 (Fig. 7). The regulation and activation of caspase-9 in the apoptosome complex is poorly understood. There are a number of possibilities to explain the reduction in the amount of cleaved caspase-9 (and subsequently the diminished enzymatic activity of caspase-9) bound to the Apaf-1 apoptosome in the SKOV3 cell line. Failure of recruitment of caspase-9 to the apoptosome could explain the phenotype seen in this cell line. Such a factor has been described (*e.g.*, Hsp70); however, our results indicate that Hsp70 is not involved in the deficient apoptosome activity seen in ovarian cancer. It is possible that another inhibiting factor that interferes with the recruitment of procaspase-9 to the apoptosome is overexpressed in ovarian cancer. Another possibility is that procaspase-9 is normally recruited to the apoptosome but is not retained. Our data demonstrates that only procaspase-9 that is recruited, activated, and retained in the apoptosome is enzymatically active. We see evidence of processed caspase-9 that has been released from the apoptosome and coelutes with the monomer form of procaspase-9 in the SKOV3 cell line (Fig. 7). Thus, another possibility is that that there may be an unknown cytosolic factor in ovarian cancer that either fails to retain activated caspase-9 in the apoptosome or that accelerates release of activated caspase-9 from the apoptosome.

In summary, our studies show that the majority of primary ovarian tumors and a subset of ovarian cell lines are deficient in Apaf-1 activity. Deficient Apaf-1 activity is linked with resistance to cisplatin-induced apoptosis in an ovarian cancer cell line. The deficient Apaf-1 activity in ovarian cancer may be attributed to a reduction in the recruitment of caspase-9 to the apoptosome or a diminished ability to retain caspase-9 in the apoptosome. This mechanism, which leads to failed apoptosis, will need to be confirmed in primary tumor samples; however, it may play an important role in resistance to chemotherapy in the clinical setting. Additional experiments to elucidate the molecular explanation for deficient Apaf-1 activation in ovarian cancer are under way.

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REFERENCES

1. Dive, C., and Wyllie, A. H. Apoptosis and cancer chemotherapy. *In:* J. A. Hickman and T. R. Tritton (eds.), Cancer Chemotherapy, pp. 21–56. Oxford, United Kingdom: Blackwell, 1993.
2. Nunez, G., Hu, Y., Benedict, M., and Inohara, N. Caspases: the proteases of the apoptotic pathway. Oncogene, *17:* 3237–3245, 1998.
3. Sun, X. M., MacFarlane, M., Zhuang, J., Wolf, B., Green, D. R., and Cohen, G. M. Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. J. Biol. Chem., *274:* 5053–5060, 1999.
4. Li, P., Nijahawan, D., Budihardjo, I., Srinivasaula, S., Ahmad, M., Alnemri, E. S., and Wang, X. Cytochrome c and dATP-dependent formation of an Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell, *91:* 479–489, 1997.
5. Green, D. R. Apoptotic pathways: the road to ruin. Cell, *94:* 695–698, 1998.
6. Yang, J., Liu, X., Bhalla, K., Kim, D. N., Ibrado, A. M., Cai, J., Peng, I. I., Jones, D. P., and Wang, X. Autoproteolytic activation of pro-caspases by oligomerization. Mol. Cell, *1:* 319–325, 1998.
7. Cain, K., Bratton, S. B., Langlais, C., Walker, G., Brown, D. G., Sun, S. M., and Cohen, G. M. Apaf-1 oligomerizes into biologically active approximately 700 kDa and inactive approximately 1.4 Mda apoptosome complexes. J. Biol. Chem., *275:* 6067–6070, 2000.
8. Rodriguez, J., and Lazebnik, Y. Caspase-9 and Apaf-1 form an active holoenzyme. Genes Dev., *13:* 3179–3184, 1999.
9. Cain, K., Brown, D. G., Langlais, C., and Cohen, G. M. Caspase activation involves the formation of the aposome, a large (~700 kDa) caspase-activating complex. J. Biol. Chem., *274:* 22686–22692, 1999.
10. Saleh, A., Srinivasula, S. M., Acharya, S., Fishel, R., and Alnemri, E. S. Cytochrome *c* and dATP-mediated oligomerization of Apaf-1 is a prerequisite for procaspase-9 activation. J. Biol. Chem., *274:* 17941–17945, 1999.
11. Soengas, M. S., Capodieci, P., Polksy, D., Mora, J., Esteller, M., Opitz-Araya, X., McCombie, R., Herman, J. G., Gerald, W. L., Lazebnik, Y., Cordon-Cardo, C., and Lowe, S. W. Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. Nature (Lond.), *409:* 207–211, 2001.
12. Beere, H. M., Wolf, B. B., Cain, K., Mosser, D. D., Mahboubi, A., Kuwana, T., Tailor, P., Morimoto, R. I., Cohen, G. M., and Green, D. R. Heat shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. Nat. Cell Biol., *2:* 469–475, 2000.
13. Pandey, P., Saleh, A., Nakazawa, A., Kumar, S., Srinivasula, S. M., Kumar, V., Weichself, R., Nalin, C., Alnemri, E. S., Kufe, D., and Kharbanda, S. Negative regulation of cytochrome c-mediated oligomerization of Apaf-1 and activation of procaspase-9 by heat shock protein 90. EMBO J., *19:* 4310–4322, 2000.
14. Merino, R., Grillot, D. A., Simonnian, P. L., Muthukkumar, S., Fanslow, W. C., Bondada, S., and Nunez, G. Modulation of anti-IgM-induced B cell apoptosis by Bcl-xL and CD40 in WEHI-231 cells. Dissociation from cell cycle arrest and dependence on the avidity of the antibody-IgM receptor interaction. J. Immunol., *155:* 3830–3838, 1995.
15. Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., and Riccadi, C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Methods, *139:* 271–279, 1991.
16. Kruk, P. A., Maines-Bandiera, S. L., and Auersperg, N. A simplified method to culture human ovarian surface epithelium. Lab. Investig., *63:* 132–136, 1990.
17. Zou, H., Henzel, W., Liu, X., Lutschg, A., and Wang, X. Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell, *90:* 405–413, 1997.

18. Benedict, M., Hu, Y., Inohara, N., and Nunez, G. Expression and functional analysis of Apaf-1 isoforms: extra WD-40 repeat is required for cytochrome c binding and regulated activation of procaspase-9. J. Biol. Chem., *275:* 8461–8468, 2000.
19. Hu, Y., Benedict, M., Ding, L., and Nunez, G. Role of cytochrome c and dATP/ATP hydrolysis in Apaf-1-mediated caspase-9 activation and apoptosis. EMBO J., *18:* 3586–3595, 1999.
20. Bratton, S. B., Walker, G., Srinivasula, S. M., Sun, X. M., Butterworth, M., Alnemri, E. S., and Cohen, G. M. Recruitment, activation, and retention of caspases-9 and -3 by Apaf-1 apoptosome and associated XIAP complexes. EMBO J., *20:* 998–1009, 2001.
21. Kuida, K., Haydar, T. F., Kuan, C-Y., Gu, Y., Taya, C., Karasujama, H., Su, M. S., Rakic, P., and Flavell, R. A. Reduced apoptosis and cytochrome c-mediated caspase-activation in mice lacking caspase-9. Cell, *94:* 325–327, 1998.
22. Hakem, R., Hakem, A., Duncan, G. S., Henderson, J. T., Woo, M., Soengas, M. S., Elia, A., de la Pompa, J. L., Kagi, D., Khoo, W., Potter, J., Yoshida, R., Kaufman, S. A., Lowe, S. W., Penninger, J. M., and Mak, T. W. Differential requirement for caspase-9 in apoptotic pathways *in vivo*. Cell, *94:* 339–352, 1998.
23. Yoshida, H., Kong, Y-Y., Yoshida, R., Elia, A. J., Hakem, A., Hakem, R., Penninger, J. M., and Mak, T. W. Apaf-1 is required for mitochondrial pathways of apoptosis and brain development. Cell, *94:* 739–750, 1998.
24. Svingen, P. A., Karp, J. E., Krajewski, S., Mesner, P. W., Gore, S. D., Burke, P. J., Reed, J. C., Lazebnik, Y. A., and Kaufmann, S. H. Evaluation of Apaf-1 and procaspases -2, -3, -7, -8, and -9 as potential prognostic markers in acute leukemia. Blood, *96:* 3922–3931, 2000.
25. Liu, J. R., Fletcher, B., Page, C., Hu, C., Nunez, G., and Baker, V. Bcl-x_L is expressed in ovarian carcinoma and modulates chemotherapy-induced apoptosis. Gynecol. Oncol., *70:* 398–403, 1998.
26. Hu, Y., Benedict, M., Wu, D., Inohara, N., and Nunez, G. Bcl-x_L interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. Proc. Natl. Acad. Sci. USA, *95:* 4386–4391, 1998.
27. Johnson, B. W., Cepero, E., and Boise, L. H. Bcl-x_L inhibits cytochrome c release but not mitochondrial depolarization during the activation of multiple death pathways by tumor necrosis factor-α. J. Biol. Chem., *275:* 31546–31553, 2000.
28. Srinivasula, S. M., Ahmad, M., Guo, Y., Zhan, Y., Lazebnik, Y., Fernandes-Alnemri, T., and Alnemri, E. S. Identification of an endogenous dominant-negative short isoform of caspase-9 that can regulate apoptosis. Cancer Res., *59:* 999–1002, 1999.
29. Hahn, C., Hirsch, B., Jahnke, D., Durkop, H., and Stein, H. Three new types of Apaf-1 in mammalian cells. Biochem. Biophys. Res. Commun., *261:* 746–749, 1999.
30. Bala, S., Oliver, H., Renault, B., Montgomery, K., Dutta, S., Rao, P., Houldsworth, J., Kucherlapati, R., Wang, X., Chaganti, R. S., and Murty, V. V. Genetic analysis of the *APAF1* gene in male germ cell tumors. Genes Chromosomes Cancer, *28:* 258–268, 2000.

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