Defective Cytochrome c-dependent Caspase Activation in Ovarian Cancer Cell Lines due to Diminished or Absent Apoptotic Protease Activating Factor-1 Activity*

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Apoptosis via the mitochondrial pathway requires release of cytochrome c into the cytosol to initiate formation of an oligomeric apoptotic protease-activating factor-1 (APAF-1) apoptosome. The apoptosome recruits and activates caspase-9, which in turn activates caspase-3 and -7, which then kill the cell by proteolysis. Because inactivation of this pathway may promote oncogenesis, we examined 10 ovarian cancer cell lines for resistance to cytochrome c-dependent caspase activation using a cell-free system. Strikingly, we found that cytosolic extracts from all cell lines had diminished cytochrome c-dependent caspase activation compared with normal ovarian epithelium extracts. The resistant cell lines expressed APAF-1 and caspase-9, -3, and -7; however, each demonstrated diminished APAF-1 activity relative to the normal ovarian epithelium cell lines. A competitive APAF-1 inhibitor may account for the diminished APAF-1 activity because we did not detect dominant APAF-1 inhibitors, altered APAF-1 isoform expression, or APAF-1 deletion, degradation, or mutation. Lack of APAF-1 activity correlated in some but not all cell lines with resistance to apoptosis. These data suggest that regulation of APAF-1 activity may be important for apoptosis regulation in some ovarian cancers.

A conserved series of events including cellular shrinkage, nuclear condensation, externalization of plasma membrane phosphatidyl serine, and oligonucleosomal DNA fragmentation characterizes apoptotic cell death. Regardless of the circumstance, induction and execution of apoptotic events require activation of caspases, a family of aspartate-specific cysteine proteinases (1). Caspase activation generally occurs via two pathways, one mediated by cell surface death receptors and the other regulated by the mitochondrion. In the death receptor pathway, death receptor ligation promotes clustering and activation of caspase-8 and -10, which in turn activate downstream caspases (reviewed in Ref. 2). By contrast, chemotherapeutic agents, UV irradiation, and other agents induce caspase activation via the mitochondrial pathway. This pathway involves mitochondrial integration of apoptotic signals and subsequent release of cytochrome c into the cytosol. The liberated cytochrome c, along with adenine nucleotides, initiates formation of an apoptosome consisting of apoptotic protease-activating factor-1 (APAF-1) oligomers. The APAF-1 apoptosome recruits and activates caspase-9, which in turn activates the executioner caspases, caspase-3 and -7 (3, 4). The active executioners kill the cell by proteolysis of key cellular substrates.

Evasion or inactivation of the mitochondrial apoptosis pathway may contribute to oncogenesis by allowing cell accumulation (5). Cell accumulation in some lymphoid malignancies, for example, involves overexpression of the antiapoptotic protein bcl-2 (reviewed in Ref. 6). bcl-2 accomplishes this by inhibiting release of mitochondrial cytochrome c, thereby promoting apoptosis resistance and cell survival (6). Recent evidence suggests that cancer cells can also attenuate caspase activity and resist apoptosis by mechanisms downstream of cytochrome c release. These include inhibition of cytosolic cytochrome c by heat shock protein (HSP)-27 (7), inactivation of APAF-1 by genetic loss or inhibition by HSP-70 and HSP-90 (8–11), and inhibition of caspases by phosphorylation or inhibitor of apoptosis proteins (12, 13). Significantly, inactivation of APAF-1 or caspase-9 by homozygous deletion enhances myc-dependent transformation, suggesting that these proteins act as tumor suppressors (14).

Ovarian epithelial cancer is the most lethal of the gynecological malignancies and affects more than 25,000 women per year (15). Ovarian cancers often resist chemotherapy-induced apoptosis (16); however, whether biochemical defects in apoptotic pathways occur in ovarian cancer is largely unknown. Here we show that ovarian cancer cell lines resist cytochrome c-dependent caspase activation in a cell-free assay, and this resistance correlates with diminished APAF-1 activity. A subset of these cell lines also resisted apoptosis, and this could be partially overcome by APAF-1 transduction. These data suggest that regulation of APAF-1 activity may be important for apoptosis regulation in some ovarian cancers.

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1 The abbreviations used are: APAF-1, apoptotic protease-activating factor-1; HSP, heat shock protein; NOE, normal ovarian epithelium; DEVD-APC, benzoyloxycarbonyl-Asp-Glu-Val-Asp-amino-4-trifluoromethyl-coumarin; MEF, murine embryonic fibroblast; JOSE, immortalized ovarian epithelial; WT, wild type; RT-PCR, reverse transcription-polymerase chain reaction.
Defective APAF-1 Activity in Ovarian Cancer Cell Lines

Fig. 1. Diminished or absent cytochrome c-dependent caspase activation in ovarian cancer cell lines. Cytoplasmic extracts were prepared from primary ovarian epithelial cells (NOE35 and NOE72), T antigen-immortalized ovarian epithelial cells (IOSE29 and IOSE50), and eight ovarian cancer cell lines. Extracts were treated with buffer or cytochrome c (10 μM) plus dATP (1 mM) for 30 min at 37 °C and then assayed for activity against the fluorogenic caspase substrate DEVD-AFC. Results represent the mean ± S.E. for at least three independent experiments.

Experimental Procedures

Materials—Granzyme B (13 units/μg) and benzoyloxycarbonyl-Asp-Glu-Val-Asp-amino-4-trifluoromethyl-coumarin (DEVD-AFC) were obtained from Enzyme Systems Products. Drs. Emad Alnemri (Thomas Jefferson University) (17), Gabriel Nunez (University of Michigan) (18), and John Reed (The Burnham Institute) (12) provided plasmids encoding caspase-9 (pRSV-LacZ-caspase-9), APAF-1 (pcDNA3-APAF-1XL), and a nonphosphorylatable caspase-9 (pCDNA3-caspase-9 S196A). pRSV-LacZ-caspase-9 was subcloned into pcDNA3 using standard techniques. Drs. Tak Mak (Amgen Institute, Ontario, Canada) and Scott Lowe (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) provided wild type (WT), APAF-1-null, and caspase-9-null murine embryonic fibroblasts (MEFs) (14, 19).

Cell Culture and Transfection—Normal ovarian epithelium (NOE) cells were isolated from normal human ovarian biopsy specimens and cultured as described previously (20). SV40 T-antigen-immortalized ovarian epithelial (IOSE) cells and ovarian cancer cell lines were derived from stocks in the Mills laboratory and originally obtained from R. Bast, N. Auersperg, and the American Type Culture Collection (20). Cells were maintained as described previously (20).

293T cells were transfected with pcDNA3-APAF-1 or pcDNA3 using the calcium phosphate method. SKOV3 cells were transfected with pcDNA3, pcDNA3-APAF-1, or pcDNA3-caspase-9 using the FuGENE 6 reagent according to the manufacturer’s instructions (Roche Molecular Biochemicals). After transfection, cells were selected for 3 weeks in media containing 0.5 mg/ml G418. The resultant G418-resistant cell populations were used for apoptosis studies and cell-free assays.

Cell-free Assays—We assessed cytochrome c/dATP-dependent caspase activation in cytosolic extracts using a cell-free assay. Reactions were initiated with 10 μM cytochrome c plus 1 mM dATP as detailed in the figure legends. After 30 min at 37 °C, DEVD-AFC was added to samples, and caspase activation was then assessed by monitoring amino-4-trifluoromethyl-coumarin release using a Tecan SpectraFluor fluorimeter in the kinetic mode (21).

In some experiments, extracts were mixed with in vitro transcribed and translated caspase-9 (22), extracts derived from MEF cell lines, or 293T cells transfected with APAF-1 or pcDNA3 before the addition of cytochrome c and dATP. We then assessed caspase activation by monitoring DEVD-AFC hydrolysis.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—Antibodies against APAF-1, actin, fodrin, poly(ADP-ribose) polymerase, and caspase-3, -7, and -9 have been described previously (22). SDS-polyacrylamide gel electrophoresis and Western blotting were performed as described previously (21).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Nucleotide Sequencing—RNA was isolated from cell lines using the guanidinium isothiocyanate-based Trizol reagent according to the manufacturer’s instructions (Life Technologies, Inc.). We then synthesized APAF-1 cDNAs by RT-PCR using oligo(dT) priming and the SuperScript system (Life Technologies, Inc.). Polymerase chain reaction products were purified by gel filtration chromatography or agarose gel electrophoresis and then sequenced using Big Dye Terminator chemistry and an ABI 7600 automated DNA sequencer. A list of the primers used in this study is available on request.

We determined the relative expression of the 12- and 13-WD repeat isoforms, APAF-1-1 isoforms by RT-PCR using the primers described by Zou et al. (4). RT-PCR products were analyzed by agarose gel electrophoresis (2% gels). This method yields 316- and 187-bp PCR products that correspond to the 13- and 12-WD repeat isoforms, respectively.

Proteasomal Degradation Assay—Whole cell extracts were treated with ATP and ubiquitin to activate the proteasome according to Li and Dou (23). We then assessed APAF-1 degradation by Western blotting after incubation of extracts at 37 °C for 1, 2, and 4 h.

Apoptosis Assays—We treated cells with cisplatin or etoposide as detailed in the figure legends and then assessed apoptosis after 48–72 h by flow cytometry using staining with annexin V and propidium iodide (21).

Fig. 2. Ovarian cancer cell lines express all cytosolic components of the apoptosome. Cytosolic extracts (50 μg of protein) were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting with antibodies against APAF-1, caspase-9, caspase-3, caspase-7, and actin. The relative cytochrome c/dATP-dependent caspase (DEV- Dase) activity for each line is indicated at the bottom of the blots. Note that the extracts displaying the four lowest DEVDase activities are italicized.
RESULTS

Diminished or Absent Cytochrome c-dependent Caspase Activation in Ovarian Cancer Cell Lines—To identify potential defects in cytochrome c/dATP-dependent caspase activation in ovarian cancer, we studied cytosolic extracts from normal and malignant ovarian epithelial cells using a cell-free system. We initiated cell-free apoptosis by adding cytochrome c (10 μM) plus dATP (1 mM) to each extract, and then we assessed caspase activation by monitoring hydrolysis of the caspase substrate DEVD-AFC (Fig. 1). NOE cells derived from two separate ovarian samples (NOE35 and NOE72) displayed the highest levels of caspase activation after cytochrome c/dATP addition. Interestingly, both T-antigen-transformed cell lines (IOSE29 and IOSE80) and all eight of the ovarian cancer lines displayed diminished cytochrome c/dATP-dependent caspase activation relative to the NOE lines. We observed profound defects in the A2780, OVCA-432, and SKOV3 cell lines with caspase activation levels less than 10% of that of the NOE cell lines. Strikingly, the OCC1 cell line had undetectable levels of cytochrome c/dATP-dependent caspase activity. Note that Fig. 1 represents the caspase activities obtained from at least three independent extract preparations per cell line, indicating that activity differences are not merely due to variations in extract preparation.

Intact Expression of Apoptosome Components in Ovarian Cancer Cell Lines—We next examined expression of apoptosis proteins in the ovarian cancer cell lines to determine whether their absence might account for the defective caspase activation observed in the cell-free assay. Fig. 2 shows that all cell lines expressed APAF-1 and caspase-3, -7, and -9 as assessed by Western blotting; however, expression levels varied significantly between the cell lines. Most cell lines expressed lower APAF-1 levels as compared with the NOE35 line. The OCC1 cell line had the most profound decrease in APAF-1 levels, expressing less than 25% of the NOE35 level. By contrast, most cell lines expressed higher levels of caspase-9 than the NOE35 cell line. We detected comparable amounts of caspase-3 in all cell lines, with the exception of the OCC1 cell line, which displayed markedly lower caspase-3 levels. Relative to the NOE35 cell line, diminished levels of caspase-7 were observed in the Hey and OCC1 cell lines, whereas the OVCA cell lines and the SKOV3 cell line expressed greater amounts of caspase-7. Even the cell lines with the lowest cytochrome c/dATP-dependent caspase activities retained expression of all apoptosis components (Fig. 2, highlighted in italic). Because we did not detect deletion of any apoptosome component, differences in APAF-1 and/or caspase activity must account for the variable levels of caspase activation observed in the cell-free assay.

Granzyme B Activates Executioner Caspases in Cytochrome c/dATP-resistant Ovarian Cancer Lines—To determine the activity of executioner caspases (caspase-3 and -7) in the cytochrome c/dATP-resistant cell lines, we added caspase activator granzyme B to each extract and monitored the generation of caspase activity. We focused on the T-antigen-transformed IOSE80 cell line and the A2780, OCC1, OVCA-432, and SKOV3 cell lines, the four cell lines with the lowest cytochrome c/dATP-dependent caspase activation (Fig. 1). Granzyme B activated DEVD-cleaving caspases in each of the resistant cell lines as shown in Fig. 3. Even the OCC1 line, which expressed relatively low levels of caspase-3 and -7 (Fig. 2), showed significant DEVDase activity with granzyme B. Similarly, granzyme B initiated DEVDase activity in the MCF7 breast cancer line, which expresses caspase-7 but lacks caspase-3. These data indicate intact executioner caspase activity in the cytochrome c/dATP-resistant cell lines and suggest that they have diminished APAF-1 and/or caspase-9 activity.

Mixing Studies Identify Defective APAF-1 Activity in the Cytochrome c/dATP-resistant Cell Lines—To determine whether the cytochrome c/dATP-resistant cell lines had defects in APAF-1 and/or caspase-9 activity, we mixed extracts from resistant cell lines with extracts from MEFs and then assessed cytochrome c/dATP-dependent caspase activation. We reasoned that mixing WT MEF extracts with the resistant extracts would restore caspase activation, provided that they did not contain dominant inhibitors of APAF-1 and/or caspase-9. We could then localize the defect to APAF-1 or caspase-9 by determining whether APAF-1-null and caspase-9-null MEF extracts could restore caspase activation. We reasoned that if the resistant cell lines had defective APAF-1 activity, then mixing the extracts with caspase-9-null MEF extract (normal APAF-1, absent caspase-9), but not the APAF-null MEF extract (absent APAF-1, normal caspase-9), would restore caspase activation. By contrast, if the resistant cell lines had defective caspase-9 activity, then mixing the extracts with the APAF-1-null MEF extract, but not the caspase-9 null extract, would restore caspase activation.

To validate this approach, we performed the experiments...
shown in Fig. 4A. The figure shows that cytochrome c and dATP initiate caspase activity in the WT MEF extract but fail to do so in the APAF-1-null and caspase-9-null extracts. However, mixing the WT extract 1:1 with the APAF-1-null extract or the caspase-null extract restores cytochrome c/dATP-dependent caspase activation. By contrast, diluting the WT extract 1:1 with buffer essentially inactivates the extract's cytochrome c/dATP-dependent caspase activation.

Fig. 4B shows the mixing experiments performed with the resistant ovarian cancer cell lines. Note that mixing the resistant cell lines with WT MEF extract restores cytochrome c/dATP-dependent caspase activation, indicating that they lack dominant inhibitors of caspase activation. Strikingly, the caspase-9-null MEF extract, but not the APAF-1-null MEF extract, partially or fully restored caspase activation in the resistant cell lines. This suggests that the resistant cell lines have defective APAF-1 activity and intact caspase-9 activity.

Supplementation Studies Confirm Defective APAF-1 Activity in the Cytochrome c/dATP-resistant Cell Lines—To confirm the preceding results, we supplemented each resistant cell line with exogenous APAF-1 or caspase-9 and then assessed cytochrome c/dATP-dependent caspase activation. As shown in Fig. 5A, the addition of APAF-1 restored caspase activation in the APAF-1-null MEF extract and in extracts from each of the
resistant cell lines. By contrast, the addition of APAF-1 to the caspase-9-null MEF extract failed to restore caspase activation. As shown in Fig. 5B, the addition of caspase-9 to the caspase-9-null MEF extract restored cytochrome c-dependent caspase activation. However, caspase-9 supplementation failed to restore caspase activation in the resistant ovarian extracts or in the APAF-1-null extract. We also supplemented the resistant extracts with a nonphosphorylatable caspase-9 mutant (S196A) because phosphorylation of caspase-9 by the serine/threonine kinase, AKT, can inhibit the activity of the proteinase (12). However, as shown in Fig. 5B, although caspase-9 S196A restored caspase activation in the caspase-9-null extract, the proteinase failed to restore caspase activation in the resistant ovarian cell lines or in the APAF-1-null extract. This suggests that caspase-9 phosphorylation does not account for the cytochrome c/dATP resistance of the ovarian cell lines.

These data confirm that the resistant ovarian cancer cell lines have intact caspase-9 activity but deficient APAF-1 activity.

Absence of APAF-1 Mutations in the Cytochrome c/dATP-resistant Ovarian Cancer Lines—We next screened the IOSE80, A2780, OCC1, OVCA-432, and SKOV3 cell lines for APAF-1 mutations because this might account for their deficient APAF-1 activity. We amplified APAF-1 cDNAs from each of the cell lines by RT-PCR and then directly sequenced the polymerase chain reaction products to assess them for APAF-1 mutations. We detected both the short (12 WD repeats) and the long (13 WD repeats) APAF-1 isoforms in each of the cell lines and sequenced each isoform in its entirety. Although we detected single-nucleotide polymorphisms in each of the cell lines, no mutations were detected. Thus, APAF-1 loss of function mutations cannot account for the resistant ovarian cancer cell lines.
cell lines defective cytochrome c/dATP-dependent caspase activation.

**APAF-1 Isoform Analysis—**Benedict et al. (18) recently showed that the 13-WD repeat APAF-1 isoform activates caspases more efficiently than the 12-WD repeat isoform. Therefore, we next determined the relative expression of each isoform in the cytochrome c-resistant ovarian cancer cell lines by RT-PCR. As shown in Fig. 6, we detected both isoforms in the NOE35 cell line and in the resistant ovarian cancer cell lines. However, in each case, the 13-WD repeat isoform was more prominently expressed. This suggests that overexpression of the 12-WD repeat APAF-1 isoform does not account for the cytochrome c resistance observed in these cell lines.

**APAF-1 Is Not a Proteasome Substrate—**Because proteasomal degradation can inactivate apoptotic proteins, we examined proteasome-dependent degradation of APAF-1. We did not detect APAF-1 degradation in whole cell extracts from the resistant cell lines even after incubation with ATP and ubiquitin for 4 h at 37 °C (data not shown).

**Apoptotic Defects in the Cytochrome c-resistant Ovarian Cell Lines—**We next examined cisplatin- and etoposide-induced apoptosis in the ovarian cell lines to determine whether their diminished APAF-1 activity attenuated chemotherapy-induced apoptosis. As shown in Fig. 7, apoptosis resistance only partially correlated with defects in APAF-1 activity. Hey cells, with relatively intact APAF-1 activity (45% DEVDase activity relative to NOE cells (Fig. 2)), were sensitive to both cisplatin- and etoposide-induced apoptosis. By contrast, the OCC1 and SKOV3 cell lines displayed low APAF-1 activity and were also resistant to both drugs. The OVCA-432 cell line was cisplatin-resistant but remained etoposide-sensitive despite diminished APAF-1 activity. Surprisingly, the A2780 cell line remained sensitive to both drugs, although it displayed low APAF-1 activity. Additionally, the IOSE80 cell line, with relatively intact APAF-1 activity, was nevertheless resistant to induction of apoptosis, presumably due to other blocks in the apoptotic pathway. Therefore, APAF-1 activity did not correlate directly with apoptosis sensitivity.

To further examine apoptosis induction in A2780 cells, we treated the cells for 48 h with 100 μM etoposide, as above. We detected caspase activation by assessment of DEVDase activity and the cleavage of the caspase substrates poly(ADP-ribose) polymerase and fodrin (data not shown). Therefore, activation of apoptosis proceeds via caspase activation in these cells despite diminished APAF-1 activity.

**APAF-1 Transfection Increases Cytochrome c-dependent Caspase Activation and Apoptosis Sensitivity in the SKOV3 Line—**We next asked whether increasing APAF-1 levels might increase the apoptosis sensitivity of resistant cells. To accomplish this, we transfected SKOV3 cells with pcDNA3-APAF-1XL, selected for populations overexpressing APAF-1, and then assessed caspase activation in vitro and cisplatin-induced apoptosis. Vector control and caspase-9-overexpressing cell populations served as controls. As shown in Fig. 8, although we obtained only 2-fold overexpression of APAF-1 in the SKOV3 cells (Fig. 8A), this overexpression restored their cytochrome c-dependent caspase activation in vitro (Fig. 8B) and enhanced their apoptosis in response to cisplatin as assessed after 72 h (Fig. 8C). Although cell death was increased in the vector-transduced control cells by this time (versus 48 h in Fig. 7), the presence of elevated APAF-1 resulted in enhanced cell death in the transduced cells. In contrast, caspase-9 overexpression failed to restore caspase activation in vitro or to enhance apoptosis in response to cisplatin.

**DISCUSSION**

The present study demonstrates that ovarian cancer cell line cytosols resist cytochrome c-dependent caspase activation. Although previous studies have shown that cancer cells can resist cytochrome c-dependent caspase activation due to modification or inhibition of caspases (12, 13), our study delineates defective APAF-1 activity as a mechanism of cytochrome c-resistance in ovarian cancer lines. Significantly, the diminished APAF-1 activity correlated with apoptosis resistance in a subset of the resistant cell lines. APAF-1 transfection could overcome this apoptosis resistance, suggesting that APAF-1 regulation may be a key mechanism for apoptosis regulation in ovarian cancer.
Recently, the phosphatidylinositol 3-kinase-Akt (protein kinase B) pathway has been implicated in ovarian carcinogenesis (20), and one way that this pathway may act is via inhibitory phosphorylation of caspase-9 (12). However, we found that cytochrome c-resistant cell lines had intact caspase-9 activity (Fig. 4) and that further supplementation with wild type caspase-9 or a nonphosphorylatable mutant (S196A) did not restore caspase activation (Fig. 5). Elevated levels of phospho (active)-Akt were not detected in the resistant ovarian extracts (data not shown), suggesting that Akt-dependent phosphorylation of caspase-9 does not limit cytochrome c-dependent caspase activation, at least under cell-free conditions. Phosphorylation or other post-translational modification of APAF-1 could potentially inhibit its activity; however, we did not detect altered APAF-1 electrophoretic mobility by SDS-polyacrylamide gel electrophoresis to suggest that it had been modified in the resistant ovarian cancer cells. Similarly, Cardone et al. (12) did not detect APAF-1 phosphorylation by metabolic labeling of colon cancer cells, suggesting that other mechanisms of APAF-1 regulation occur in cancer cells.

Losses of function mutations destroy enzymatic activity and may represent another means for APAF-1 inactivation. However, our study shows expression of only wild type APAF-1 in the five resistant lines examined. Similarly, others (8, 24, 25) have shown a lack of APAF-1 mutations in a series of malignant tumors including melanomas, lymphomas, teratomas, germ cell tumors, and pancreatic, cervical, and colorectal carcinomas. Although Yamamoto et al. (26) demonstrated APAF-1 mutations in colorectal cancer; this was limited to the subset of tumors exhibiting defects in mismatch repair genes (i.e. the microsatellite mutator phenotype). Because the microsatellite mutator phenotype is not common in ovarian cancer (27), ovarian cancers are not likely to acquire APAF-1 mutations from this mechanism. Besides mutation, altered splicing of APAF-1 influences the protein’s activity, with isoforms expressing 12 WD repeats demonstrating diminished cytochrome c/dATP-dependent caspase-activating activity (18). However, we detected both 12- and 13-WD repeat APAF-1 isoforms, indicating that restricted isoform expression does not account for diminished APAF-1 activity in ovarian cancer cell lines.

Degradation or inhibition of apoptotic proteins can promote apoptosis resistance. Proteosomal degradation of apoptotic proteins represents one mechanism for their inactivation (23); however, we found that proteosomal degradation of APAF-1 does not occur in the resistant ovarian lines, suggesting a potential role for APAF-1 inhibitors. Because we could restore APAF-1 activity in the cytochrome c-resistant ovarian extracts by mixing them with MEF extracts (Fig. 4) or by adding exogenous APAF-1 (Fig. 5), our data suggest the presence of a nondominant (i.e. competitive) APAF-1 inhibitor. Recently, we (10) and others (9) have identified HSPs as inhibitors of APAF-1 activity, and in the case of HSP-70, APAF-1 inhibition occurs in a nondominant fashion. Because induction of HSPs occurs during stress responses and cancer (28), investigation of potential interactions between APAF-1 and HSPs in ovarian cancer is an important topic for future investigation.

Despite diminished APAF-1 activity, apoptosis still proceeded in two of the cell lines, A2780 and OVCA-432 (Fig. 7). In A2780, this apoptosis correlated with increased DEVase activity and the cleavage of two caspase substrates, poly(ADP-ribose) polymerase and fodrin, in the expected pattern for caspase cleavage (data not shown). Either the existing low level of APAF-1 activity was still sufficient to drive caspase activation in these cells or alternative pathways for apoptosis were engaged. APAF-1-independent caspase activation and apoptosis in multiple myeloma cells were recently suggested in another study, and it was proposed that these events occurred via activation of caspasases by the release of Smac/DIABLO from mitochondria (30). Alternatively, DNA damage can induce the expression of Fas (CD95) ligand in a T-cell lymphoma cell line (31) and a colon carcinoma line (32), leading to engagement of Fas (CD95) and caspase activation. Apoptosis via Fas or other death receptors can proceed independently of APAF-1 (19).

Deletion or silencing of tumor suppressor genes can abolish expression of their protein products and thereby promote oncogenesis. Although Soengas et al. (8) implicated APAF-1 as a tumor suppressor by demonstrating its deletion in metastatic melanoma, we (Fig. 2) and others (18, 24, 25) have not identified APAF-1 deletion in various other cancer cell lines including lines from leukemias, lymphomas, teratomas, germ cell tumors, and pancreatic, cervical, ovarian, and colorectal carcinomas. This suggests that APAF-1 is not a classical tumor suppressor or that other mechanisms exist to abolish APAF-1 activity. Significantly, our study shows that ovarian cancer cell lines generally demonstrate diminished or absent APAF-1 activity compared with NOE cell lines (Fig. 1). This decreased APAF-1 activity correlated in many cases with decreased APAF-1 expression relative to the NOE cell lines (Fig. 2). Furthermore, supplementing cell lines defective in APAF-1 activity restored their cytochrome c-dependent caspase activation (Figs. 4 and 5), suggesting that APAF-1 concentration is an important determinant of its activity. Interestingly, overexpression of APAF-1 (but not caspase-9) in SKOV3 cells enhanced their apoptosis in response to cisplatin (Fig. 8). Because similar apoptosis sensitization was seen with leukemia and melanoma cells (8, 29), APAF-1 levels may modulate the apoptotic threshold of cancer cells. Therefore, factors that increase APAF-1 levels or enhance APAF-1 activity may hold promise as therapeutic agents for cancer.

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