[CANCER RESEARCH 64, 696–703, January 15, 2004]

Resveratrol-induced Autophagocytosis in Ovarian Cancer Cells

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ABSTRACT

Resveratrol (3,5,4-trihydroxystilbene), a natural phytoalexin present in grapes, nuts, and red wine, has antineoplastic activities. Several molecular mechanisms have been described to underlie its effects on cells *in vitro* and *in vivo*. In the present study, the response of ovarian cancer cells to resveratrol is explored. Resveratrol inhibited growth and induced death in a panel of five human ovarian carcinoma cell lines. The response was associated with mitochondrial release of cytochrome *c*, formation of the apoptosome complex, and caspase activation. Surprisingly, even with these molecular features of apoptosis, analysis of resveratrol-treated cells by light and electron microscopy revealed morphology and ultrastructural changes indicative of autophagocytic, rather than apoptotic, death. This suggests that resveratrol can induce cell death through two distinct pathways. Consistent with resveratrol's ability to kill cells via nonapoptotic processes, cells transfected to express high levels of the antiapoptotic proteins Bcl-x_L and Bcl-2 are equally sensitive as control cells to resveratrol. Together, these findings show that resveratrol induces cell death in ovarian cancer cells through a mechanism distinct from apoptosis, therefore suggesting that it may provide leverage to treat ovarian cancer that is chemoresistant on the basis of ineffective apoptosis.

INTRODUCTION

In the United States, epithelial ovarian carcinoma is the leading cause of death among patients with gynecological cancers. It is the fifth most frequent cause of cancer death in women, and 15,000 women die of this disease annually (1). Although the majority of tumors initially respond to chemotherapy, unfortunately, most patients succumb to recurrent tumors that are composed of chemotherapy-resistant clones. Despite improved survival for many malignancies during the recent era in clinical oncology, the 5-year survival for patients with advanced stage ovarian cancer has remained unchanged (<20%) over the past 20 years (2).

Resistance of recurrent disease to cytotoxic drugs is the principal factor limiting long-term treatment success against ovarian cancer. The oncogenesis of ovarian cancer in particular appears to favor the development and subsequent expansion of cell clones that are resistant to apoptotic triggers. The basis for failed apoptosis and more specifically the cause of chemotherapy resistance in ovarian cancer is multifactorial. Molecular mechanisms implicated to date include expression of P-glycoproteins (*e.g.* multidrug resistance pumps), p53 mutations, and high-level expression of Bcl-2 and other inhibitors of apoptosis that block caspases and stabilize the mitochondrial permeability pore (3–5). Therefore, an important research objective is the identification of lead compounds that circumvent the resistance mechanisms that limit the success of conventional drugs.

Resveratrol (3,4′-trihydroxystilbene), a natural product from grapes that is present in significant concentrations in red wine, inhibits *in vitro* cell growth of leukemias, prostate, breast, and other epithelial cancers (6–13). Moreover, resveratrol blocks the development of preneoplastic lesions in carcinogen-treated mammary glands in culture (14). In animal models of carcinogenesis, experiments to evaluate resveratrol's chemopreventive properties have been similarly encouraging. In mice, resveratrol blocks tumorigenesis in skin damaged by UV radiation, and in rats it blocks mammary gland neoplasia (8, 15).

Resveratrol has multiple molecular and biochemical effects: it produces antioxidant and pro-oxidant effects; it directly binds to several molecular targets including estrogen receptors and the F1 component of mitochondrial ATP synthase; it inhibits tubulin polymerization; and it induces cell cycle arrest and apoptosis (6, 9–11, 15–17). Resveratrol's antiproliferative effects are associated with changes in the expression or phosphorylation of cyclin E, cyclin A, cdc2, and Rb, and most commonly, causes cell cycle arrest at the G_2-S checkpoint (10, 11, 13, 18). Resveratrol-induced apoptosis appears to involve caspase activation and stabilization of p53 (12, 13, 19). Thus, multiple overlapping mechanisms can contribute to the compound's activity against precancerous or cancer cells.

The purpose of this work was to develop an understanding of resveratrol's effects on ovarian cancer cells to begin to determine its therapeutic value in preventing or treating this disease. In this report, we describe experiments that show resveratrol induces programmed cell death in ovarian cancer cells. The death mechanism was characterized, revealing that resveratrol not only initiated apoptosis but ultimately caused cell death through autophagocytosis (type II programmed cell death). In an important model of chemotherapy resistance, resveratrol's cytotoxic effects were not limited by high-level expression of either Bcl-2 or Bcl-x_L, both of which significantly reduce the response to standard chemotherapeutic agents, including cisplatin. Together, these results identify ovarian cancer as a potential therapeutic area to consider for resveratrol. Moreover, autophagocytosis is an additional biological mechanism that accounts for the actions of this compound.

MATERIALS AND METHODS

Chemicals. Resveratrol, monodansylcadaverine (MDC), and 3-methyladenine (3-MA) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Resveratrol was dissolved in aqueous DMSO and delivered to cells in media containing this solvent at a final concentration of 0.1% (v/v). Benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk), benzyloxycarbonyl-Leu-Glu-Thr-Asp-fluoromethyl ketone (z-LETD-fmk), and benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethyl ketone (z-LEHD-fmk) were obtained from Enzymes Systems Products (Livermore, CA).

Cell Lines, Plasmids, and Immunoblotting. Ovarian cancer cell lines A2780 and CaOV3 were obtained from the American Type Culture Collection (Manassas, VA). Dr. K. Cho (University of Michigan) generously provided ES-2, TOV112D, and A1947 cell lines. Ovarian cancer cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin (Life Technologies, Inc., Carlsbad, CA). A2780 cells were transfected with pcDNA3-Bcl-x_L, control pcDNA3 plasmid, pSFFVneo-Bcl-2, or control pSFFVneo plasmid, using Lipofectamine (Life Technologies, Inc.). Individual cell clones were selected for growth in the presence of G418 (500 μg/ml; Bio-Rad Laboratories, Hercules, CA) by limiting dilution. Expression of Bcl-x_L and Bcl-2 in single-cell clones was analyzed by immunoblotting. Cytosolic extracts were prepared as described (20). Apaf-1 was detected with a monoclonal antibody obtained from Trevigen (Gaithersburg, MD). Monoclonal antibody for caspase-9 was obtained from

Received 8/4/03; revised 11/4/03; accepted 11/5/03.

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Stressgen (San Diego, CA). The monoclonal antibody for Bcl-x_L was obtained from BD Transduction (Franklin Lakes, NJ). The monoclonal antibody for cytochrome *c* and polyclonal antibody for caspase-3 were obtained from BD PharMingen (San Diego, CA). The monoclonal antibody for β-tubulin was obtained from Sigma-Aldrich. The monoclonal antibody for cytochrome oxidase subunit IV was obtained from Molecular Probes (Eugene, OR). Western blots were developed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). Densitometry analysis was performed using Scion Image β4.02 software from Scion Corp. (Frederick, MD).

Subcellular Fractionation. For the preparation of subcellular fractions, cells were harvested, washed with cold PBS, suspended in 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] and homogenized with 10 strokes with a Dounce homogenizer. Nuclei were pelleted at 1,000 × *g* for 10 min (4°C). The mitochondrial fraction was harvested by centrifugation at 10,000 × *g* for 30 min (4°C). The supernatant was harvested as the cytoplasmic fraction (S-100 extract). The purity of fractions was tested by immunoblotting with antibodies specific for either β-tubulin (cytoplasmic protein) or cytochrome *c* oxidase (mitochondria membrane protein).

Analysis of Viability and Apoptosis. Cells were plated, and 24 h after plating, 50–200 μM resveratrol was added to the culture medium. The percentage of apoptotic cells was determined at the indicated time points by propidium iodide (PI) staining on semi-permeabilized cells as described previously (21). In this assay, apoptotic cells are identified on the basis of hypodiploid DNA content that results from DNA fragmentation (22). Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining was performed using the Apoptosis Detection System kit (Promega, Madison, WI). To assess viability, intact cells were harvested, stained with PI, and evaluated by flow cytometry for plasma membrane permeability to PI.

Fractionation of Cytosolic Extracts by Gel Filtration. A2780 cells were treated with resveratrol (50 μM) or vehicle control (DMSO, 0.1%) for 24 h, and S-100 cytosolic extracts were prepared as described above. Two mg of S-100 extracts were loaded on a Superdex-200 HR gel filtration column (Amersham Pharmacia Biotech, Piscataway, NJ) pre-equilibrated with buffer A (see above) at a flow rate of 0.5 ml/min using a Bio-Rad BioLogic HR Workstation. The column was calibrated with Amersham Pharmacia Biotech HMW gel filtration standards (thyroglobulin, M_r 669,000; ferritin, M_r 440,000; catalase, M_r 232,000; BSA, M_r 66,000). After discarding the majority of the void volume, 400-μl fractions were collected. Aliquots (50 μl) of each fraction were resolved by SDS-PAGE and immunoblotted to detect Apaf-1 and caspase-9.

Ultrastructural Characterization. For transmission electron microscopy (TEM), treated cell monolayers were rinsed with 0.1 M Sorensen's buffer (pH 7.4) and then fixed for 1 h in glutaraldehyde (2.5% w/v) in the same buffer. After a buffer rinse, they were postfixed for 15 min in osmium tetroxide (1% w/v), rinsed in water, and then *en block* stained for 30 min in aqueous uranyl acetate (3% w/v). Cell monolayers were then scraped and pelleted, dehydrated in a graded series of ethanol baths, then infiltrated and embedded in Spurr's resin. Ultrathin sections were poststained with uranyl acetate and lead citrate and viewed on a Philips CM100 at 60 kV.

Fluorescence Microscopy. Hoechst staining was performed to evaluate nuclear morphology. Cells were incubated with Hoechst 33258 (5 μg/ml) for 15 min at ambient temperature. Wet-mounts were visualized by a combination of differential interference contrast and fluorescence microscopy (Leica DMLB, Wetzlar, Germany). Images were captured using a using a SPOT RT camera (Diagnostic Instruments Inc., Sterling Heights, MI).

Autophagic vacuoles were specifically identified using MDC. Cells, growing on glass coverslips, were treated with resveratrol or vehicle control as indicated, and after treatment, the medium was exchanged to contain 50 μM MDC in PBS and incubated for 10 min at 37°C. After incubation, coverslips were washed four times with PBS and immediately analyzed by microscopy to detect fluorescent staining by MDC (Leica DMLB). Images were captured using a SPOT RT digital camera (Diagnostic Instruments).

Statistical Analysis. When appropriate, statistical significance was tested using a two tailed Student's *t* test; $P \leq 0.05$ was considered significant. All values shown are means with the corresponding SE.

RESULTS

Resveratrol Induces Cell Death and Growth Inhibition in Ovarian Cancer Cells. Treating A2780 cells with resveratrol resulted in cell death by 24 h (Fig. 1*A*). Proliferation of resveratrol-treated A2780 cultures were analyzed over 72 h of continuous treatment during which time there was no further growth (Fig. 1*B*). To determine whether the cytotoxic response to resveratrol was unique to A2780 cells, four additional ovarian cancer cell lines were tested. Resveratrol induced cell death in each line (Fig. 1*C*). Interestingly, in experiments designed to measure viability with flow cytometry, we found that resveratrol caused an unexpected, pronounced shift in the forward-side scatter parameters of A2780 and CaOV3 cells (Fig. 1*D*). These changes in light scatter suggested that treatment caused cells to become larger (increase in forward scatter) and more complex (increase in side scatter). This was surprising because apoptotic death is typically associated with rapid cell shrinkage (23).

Resveratrol Affects Cycle Progression and Blocks Proliferation. Although it was apparent that resveratrol was cytotoxic to A2780 and other ovarian cell lines, the sustained effects on culture growth raised suspicion that it might also be affecting the proliferation of surviving cells. To test this possibility, we analyzed whether resveratrol affects cell cycle progression. As shown in Fig. 2*A*, within 24 h, at a concentration of 50 μM, resveratrol caused cells to accumulate in S-phase. At 100 μM, the majority of cells instead accumulated in G_0-G_1 (Fig. 2*A*). Interestingly, we did not detect an increase in cells with hypodiploid DNA content, suggesting that apoptotic cell death was not induced. Consistent with our data, a concentration dependence to resveratrol's effects on the cell cycle has been reported previously (10, 18, 24, 25).

To determine the reversibility of the growth arrest caused by resveratrol, A2780 cells were incubated with resveratrol (50 μM) for increasing amounts of time (0–48 h), after which the drug was

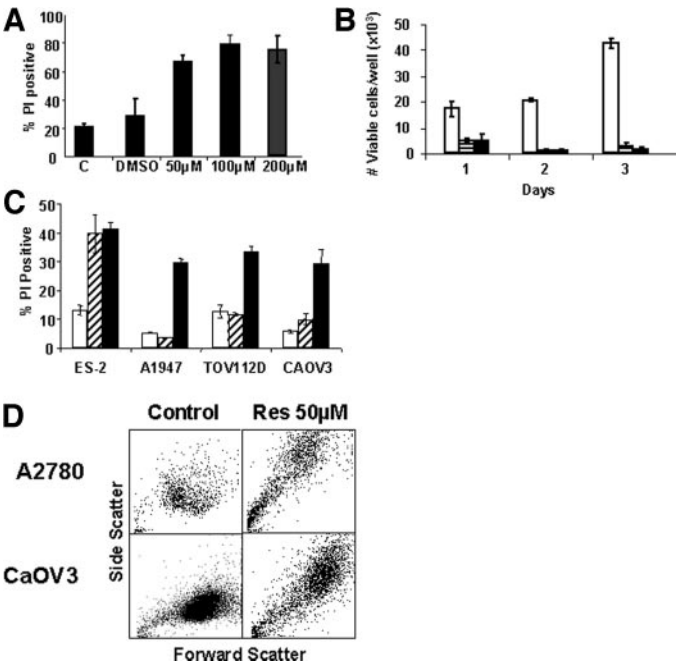

Fig. 1. Resveratrol-induced cell death and growth inhibition in ovarian cancer cells. *A*, dose-dependent killing of A2780 cells with resveratrol. Viability after 24 h was determined by PI exclusion. *B*, A2780 cell proliferation after treatment with vehicle control (□, DMSO), 25 μM resveratrol (▤), 50 μM resveratrol (■). Viable cells were quantified by trypan blue staining. *C*, cell death determined after 24 h (▨) and 48 h (■) of exposure to resveratrol (50 μM) or to vehicle control (48 h only, □) for the indicated cell lines. In *D*, the forward-side scatter profiles of resveratrol-treated ovarian cells are shown. Data for *A*, *B*, and *C* are presented as means; *bars*, SD.

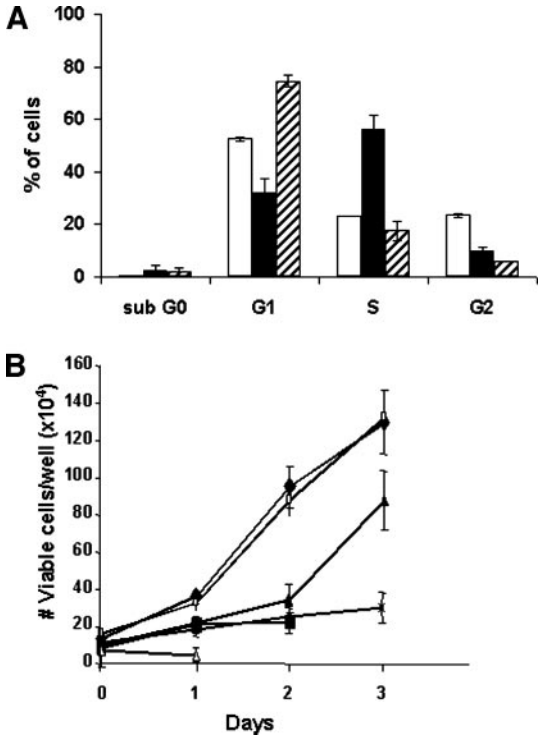

Fig. 2. Effect of resveratrol on cell cycle distribution. *A*, cell cycle distribution from cultures treated for 24 h with vehicle control (□), resveratrol (50 μM; ■), or resveratrol (100 μM; ▨). Cell cycle assignment was based on DNA content detected with PI by flow cytometry and is representative of three or more experiments. *B*, A2780 cells were treated with resveratrol (50 μM) and at 0 h (♦), 6 h (□), 12 h (▲), 24 h (×), 36 h (■), and 48 h (△), cells were washed, and cell number was monitored for 3 days. Viable cell number was determined by cell counting, and the data are presented as means; *bars*, SD.

removed, and the viable cells were replated at a constant density in fresh medium without resveratrol. As shown in Fig. 2*B*, treating with resveratrol for >12 h causes irreversible growth arrest. Together, these results demonstrated that resveratrol induces irreversible growth inhibition that is associated with specific effects on the cell cycle and ultimately results in cell death.

Resveratrol Induced Cell Death Is Not Inhibited by Bcl-2 or Bcl-x_L. In certain cell types, antiapoptotic proteins, including Bcl-2, attenuate resveratrol-induced cell death (26). Given the significance of Bcl-2 and Bcl-x_L as mediators of chemotherapy resistance in ovarian cancer, we used single gene transfection to determine whether either protein inhibits resveratrol induced killing. A2780 clones, stably transfected to express high levels of Bcl-2 or Bcl-x_L (Fig. 3*A*), were compared with vector controls to determine responsiveness (*i.e.*, affect on viability) to resveratrol and cisplatin. In these experiments, each agent was used at a concentration that kills 50% of the parental cells. As seen in Fig. 3*B*, the degree of resveratrol-induced cell death in control cells (50%) was comparable with that seen in Bcl-2 (35%) and Bcl-x_L (40%) expressing cells ($P \geq 0.05$). In contrast, cisplatin induced cell death in 51% of control cells as compared with only 16.5 and 16.3% in Bcl-2 and Bcl-x_L expressing cells ($P \leq 0.05$). These results indicate that apoptotic pathways controlled by Bcl-2 and Bcl-x_L are not important for resveratrol-induced cell death in A2780 cells. This conclusion also suggests that nonapoptotic pathways mediate the response to this drug, consistent with the earlier findings that hypodiploid DNA changes were not detected in resveratrol-treated cells (see Fig. 2*A*).

Resveratrol Causes Cytochrome *c* Release from Mitochondria. Mitochondria play an essential role in apoptosis and necrosis triggered by chemical agents. The mitochondria response includes release of cytochrome *c* into the cytosol. In the cytosol, cytochrome *c* binds to Apaf-1, allowing recruitment of caspase-9 and formation of the apoptosome, resulting in caspase activation and execution of cell death (27–30). To determine whether resveratrol affects this death mechanism, we tested whether it triggers release of cytochrome *c* from the mitochondria. After treatment for 24 h with vehicle control, resveratrol, or cisplatin (a positive control), A2780 cells were fractionated into separate cytosol and mitochondrial fractions. The purity of fractions was verified by the presence of β-tubulin and cytochrome oxidase exclusively in the cytosolic and mitochondrial fractions, respectively (Fig. 4). As seen in *Lanes 3* and *4* of this figure, cytochrome *c* is only present in the mitochondrial fraction of vehicle control cells. However, the protein was detected in the cytosol, as well as mitochondria, of resveratrol and cisplatin-treated cells, indicating that it is released from mitochondria during treatment. Resveratrol-induced release of cytochrome *c* was at most partially inhibited by z-VAD-fmk (Fig. 4, *Lanes 9–12*), indicating that this response is independent of caspase activity.

Resveratrol-induced Apoptosome Activation Is Not Required for Cell Death. To determine whether resveratrol treatment results in formation of the Apaf-1/caspase-9 apoptosome, we used size exclu-

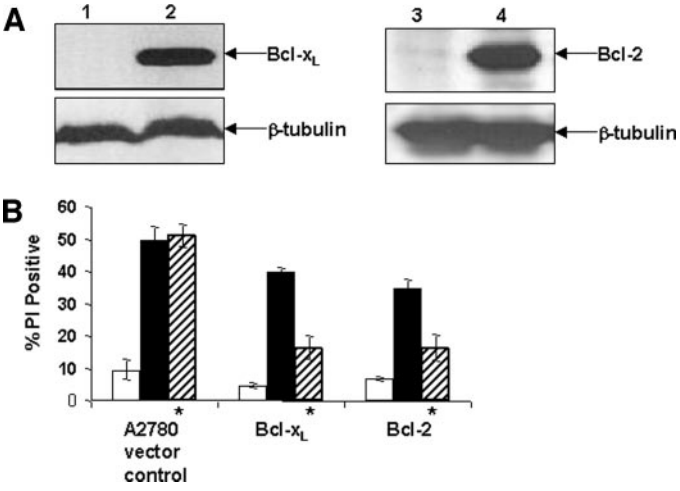

Fig. 3. Resveratrol-induced cell death is not blocked by overexpression of Bcl-2 or Bcl-x_L. *A*, A2780 cells stably transfected to express Bcl-2 (in pSFFVneo) and Bcl-x_L(in pcDNA3). Unique transfected clones were isolated, and expression of proteins was verified by immunoblotting. *Lane 1*, A2780-pcDNA3; *Lane 2*, A2780-Bcl-x_L; *Lane 3*, A2780-pSFVneo; *Lane 4*, A2780-Bcl-2. *B*, cell death determined after 24 h of exposure to vehicle control (□), resveratrol (50 μM; [squlf]), or cisplatin (3 μM; ▨) of A2780 and overexpressing clones. *, statistical significance ($P \leq 0.05$). Data represent the means; *bars*, SD.

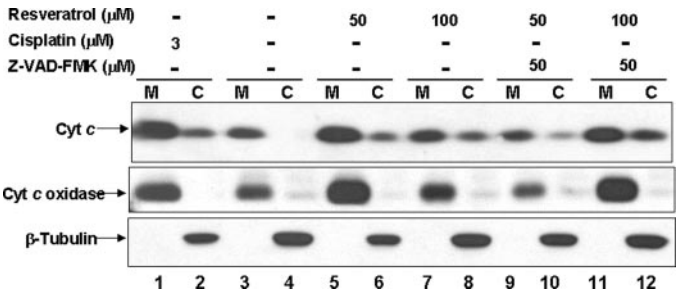

Fig. 4. Resveratrol-induced apoptosis results in the release of mitochondrial cytochrome *c*. A2780 cells were treated with cisplatin (3 μM; *Lanes 1* and *2*), DMSO (*Lanes 3* and *4*), and resveratrol (50 and 100 μM) in the presence or absence of z-VAD-fmk as indicated for 24 h (*Lanes 5–12*). Cells were harvested and separated into cytosolic (*C*) and mitochondria (*M*)-enriched fractions. Thirty μg of protein were resolved by SDS-PAGE and immunoblotted to detect cytochrome *c* (*Cyt c*). The purity of the cytosolic and mitochondrial enriched fractions was confirmed with monoclonal antibodies for cytochrome *c* oxidase, subunit IV, and β-tubulin.

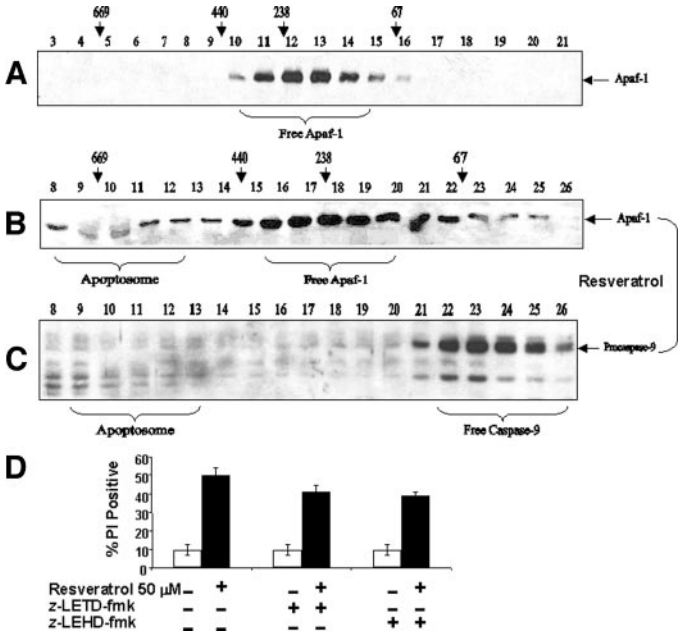

Fig. 5. Apoptosome formation after treatment with resveratrol. *A,* S100 cytosolic lysates were prepared from A2780 cells. Lysates were then fractionated by fast protein liquid chromatography over a Superdex 200 HR column. Aliquots were resolved by SDS-PAGE, and Apaf-1 was detected by immunoblotting. The elution profiles with selected size standards are indicated. *B* and *C,* cytosolic S100 samples were isolated from A2780 cells after treatment with 50 μM resveratrol for 24 h and size-fractionated as indicated above. Immunoblotting for Apaf-1 **(***B***)** and caspase-9 **(***C***)** was performed. *D,* A2780 cells were treated with 50 μM resveratrol for 24 h in the absence or presence of z-LETD-fmk (an irreversible caspase-8 inhibitor) or z-LEHD-fmk (an irreversible caspase-9 inhibitor). Cells were then analyzed for viability (membrane permeability to PI) by flow cytometry. Data represent the means; *bars,* SD.

sion chromatography to fractionate native cytosolic extracts from treated cells on the basis of molecular weight. Individual fractions from gel filtration chromatography were then analyzed for Apaf-1 and caspase-9 by immunoblotting. In the absence of resveratrol, Apaf-1 eluted in fractions consistent with it being present exclusively as a monomer (fractions 10–15, Fig. 5*A*). In cells treated with resveratrol, Apaf-1 and caspase-9 were present in high molecular weight fractions consistent with their presence within the ~700 kDa to 1.4 MDa apoptosome complex (fractions 8–13, Fig. 5, *B* and *C*). Apoptosome complex-associated caspase-9 was processed, being detected as its catalytically active subunits (p35/p37). Fractions 21–25 from treated cells contained procaspase-9 and cleaved caspase-9, which was apparently released from the Apaf-1 apoptosome.

To determine whether caspase-9 activity is necessary for resveratrol killing, cells were incubated with either z-LETD-fmk (as a negative control) or z-LEHD-fmk, inhibitors of caspases -8 and -9, respectively, before resveratrol treatment. As seen in Fig. 5*D*, neither caspase inhibitor significantly reduced killing by resveratrol: resveratrol alone induced killing in 50% of cells, as compared with 41 and 39% in cells treated with resveratrol plus LETD or LEHD ($P \geq 0.05$). These results suggest that although caspase-9 is activated, it is not required for resveratrol-induced cell death. Moreover, identical results were obtained using the pan-caspase inhibitor z-VAD-fmk (data not shown), indicating that caspase activity, in general, is not necessary for A2780 cell death in response to resveratrol.

Resveratrol Induces Autophagocytosis. Despite cytochrome *c* release, apoptosome formation, and caspase-9 cleavage, the majority of resveratrol-treated A2780 cells do not display morphology typical of apoptosis. Resveratrol-induced cell death occurs independently of caspase activity, is not inhibited by Bcl-2 or Bcl-x_L, and does not result in hypodiploid DNA content (see Fig. 2*A*). Moreover, as seen in Fig. 6, the majority of resveratrol-treated cells do not display morphology consistent with apoptosis. In particular, A2780 cells respond to 50 μM resveratrol, a concentration that kills 50–75% of cells, without cytoplasmic condensation, nuclear fragmentation, or blebbing (Fig. 6*C*), features typical of apoptosis (23). A2780 cell death induced by resveratrol is associated with a microscopic appearance different from the appearance of cells treated with 3 μM cisplatin, a concentration that also kills 50–75% of A2780 cells, with the expected morphological features of apoptosis (Fig. 6, compare *C* with *B*). Resveratrol-treated cells do not condense, nuclei remain intact, and the cytoplasm and cell surface develop a highly granular appearance. Bcl-x_L-expressing clones are resistant to cisplatin-induced apoptosis as compared with control cells, although there are some typically apoptotic cells present after cisplatin treatment (Fig. 6, *E* compared to *D*). In contrast, Bcl-x_L-expressing clones treated with resveratrol are similar in morphology to resveratrol-treated A2780-pcDNA3 cells, with a highly granular appearance to the cytoplasm and no evidence of apoptosis (Fig. 6, *F* and *C*). Overall, these morphological characteristics provide further support for the hypothesis that nonapoptotic death mechanisms can mediate the response to resveratrol.

TUNEL staining, a standard technique commonly applied to detect apoptosis, was performed on resveratrol (50 μM)-treated cells after 24 and 48 h. Resveratrol caused unequivocally positive TUNEL staining (data not shown). However, because transcription (31, 32), artificial DNA breakage, necrosis (33), and nonapoptotic programmed death (34, 35) each lead to DNA changes that give positive results on TUNEL, these findings were not helpful in further defining the mechanism of cell death. Electron microscopic characterization, which has been the gold standard for most precisely determining the mode of cell death, was next used to distinguish between these possibilities.

Nonapoptotic programmed cell death is principally attributed to autophagy (type II programmed cell death). Autophagy is a series of biochemical steps through which eukaryotic cells commit suicide by degrading their own cytoplasm and organelles through a process in which these components are engulfed and then digested in double membrane-bound vacuoles called autophagosomes (36). Examination of control cells using TEM revealed normal nuclear and mitochondrial morphology (Fig. 7, *A* and *B*). Resveratrol treatment resulted in the appearance of autophagocytic granules by 24 h (Fig. 7, *C* and *D*). After 48 h of resveratrol treatment, cells undergoing autophagic cell death retained an intact nuclear membrane, without chromatin condensation. Autophagocytic granules contained extensively degraded organelles (Fig. 7, *E* and *F*). Cells treated with cisplatin showed

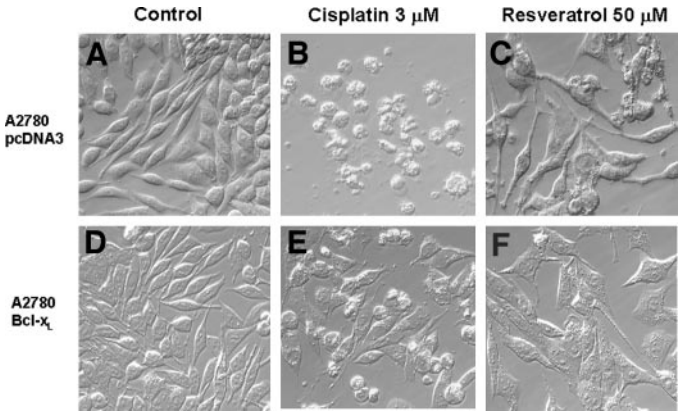

Fig. 6. Morphology of resveratrol-treated ovarian cancer cells. The morphologies of A2780 (*A–C*) and A2780-Bcl-x_L-expressing cells (*D–F*) treated with DMSO (*A* and *D*), cisplatin (3 μM; *B* and *E*), and resveratrol (50 μM; *C* and *F*) were determined by interference light microscopy.

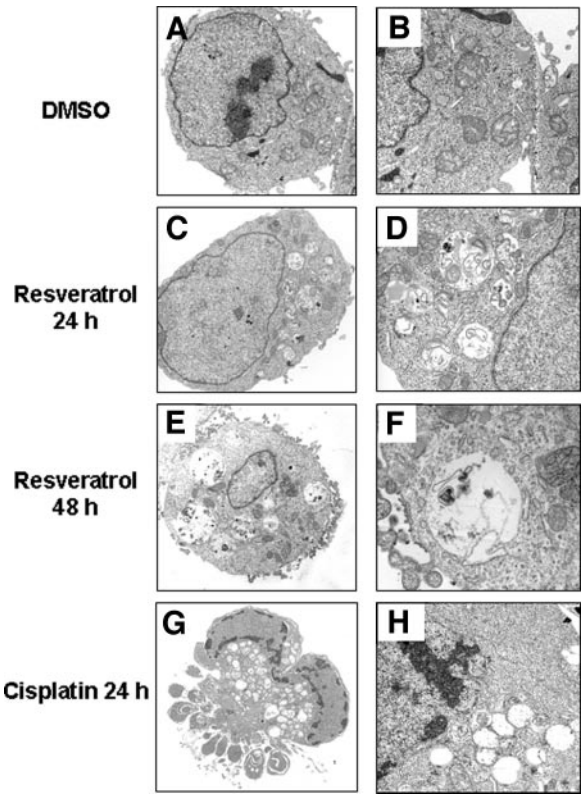

Fig. 7. Resveratrol induces autophagy in ovarian cancer cells. Cells were examined using TEM. Nuclear and mitochondrial morphologies are normal in control cells (*A*, ×4600; *B*, close-up of mitochondria). Resveratrol treatment results in autophagocytic granules by 24 h (*C*, ×4600; *D*, close-up of autophagosome showing membrane-wrapped organelles). Late-stage autophagy in cells after 48 h of resveratrol demonstrates an intact nuclear membrane, chromatin is not condensed (*E*, ×4600; *F*, close-up of autophagosome with extensively degraded organelles). Cells treated with cisplatin show characteristics of apoptosis (*G*, chromatin condensation, blebbing of apoptotic bodies, ×4600; *H*, close-up of mitochondrial swelling). Note the absence of autophagocytic bodies in *G* and *H*.

characteristic changes associated with apoptosis, including intact cellular membranes, blebs at the cellular surface consistent with the formation of apoptotic bodies, and chromatin condensation, in the absence of autophagocytic bodies (Fig. 7, *G* and *H*).

To confirm that resveratrol-induced cell death was largely nonapoptotic, nuclear morphology was evaluated using Hoechst staining. A2780 ovarian cancer cells were treated with DMSO solvent control, resveratrol, or cisplatin. The nuclei of resveratrol-treated cells appeared similar to control DMSO-treated cells. In contrast, cisplatin treatment resulted in the appearance of clumped, condensed chromatin, along with a disintegrating nuclear envelope (Fig. 8, *A–C*). MDC, a fluorescent compound selectively taken up by autophagosomes, was used to obtain independent evidence supporting the conclusion that resveratrol triggers autophagocytosis (37). MDC was applied to A2780 cells after resveratrol treatment, and when these labeled cells were imaged using epifluorescence microscopy, treated cells demonstrated an intense, punctate fluorescence pattern (Fig. 8*E*). In contrast, control cells had minimal fluorescence (Fig. 8*D*). To further implicate autophagocytosis, we planned to pretreat A2780 cells with the phosphoinositide kinase inhibitor, 3-MA, known to block autophagocytic signaling (38). Unfortunately, appropriate concentrations of 3-MA *alone* killed these cells, precluding the use of this agent as intended (data not shown).

DISCUSSION

Resveratrol is a phytoalexin that helps protect plants against pathogens (39). Specific anticancer effects of resveratrol have also been shown *in vitro* and *in vivo*. The most persuasive evidence shows that resveratrol affects early steps in the process of carcinogenesis. It inhibits the formation of neoplastic skin and mammary lesions (14, 40), it blocks transformation of rat epithelial cells by *N*-nitrosomethylbenzylamine (41), and it suppresses gastrointestinal tumor formation in *Min* mice (42).

Resveratrol may also limit the survival and proliferation of cancer cells, more consistent with the actions of a conventional chemotherapeutic drug. Its influence on cellular redox balance, inhibitory effects on estrogen hormone signaling, and antiangiogenic functions may all be relevant for its effects on the late stages of carcinogenesis. Resveratrol's antiproliferative and apoptosis-inducing capabilities have been demonstrated in many cell types (43). Its biochemical actions are coupled to multiple signaling pathways, such as nuclear factor-κB, cyclo-oxygenase-2, and inducible nitric oxide synthase expression, and it changes the levels of several components of the cyclin-dependent kinase system (44–48). Nevertheless, outside of a few early reports, there is disappointingly little conclusive evidence that demonstrates its effectiveness at treating already existing tumors or malignancies in animals (8, 49, 50).

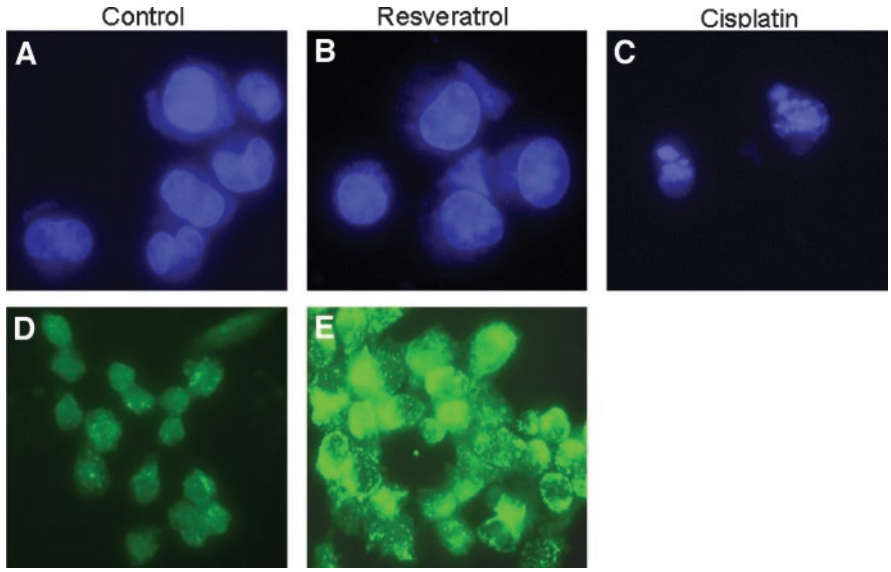

Fig. 8. Resveratrol-treated cells exhibit nonapoptotic nuclear morphology and cytoplasmic autophagosomes. A2780 cells were treated with vehicle control (*A*), 50 μM resveratrol (*B*), or 3 μM cisplatin (*C*) for 24 h. Nuclear morphology was evaluated by Hoecsht staining. Autophagosomes were labeled using MDC in A2780 cells treated with vehicle control (*D*) or 50 μM resveratrol (*E*) for 24 h. An intense, granular pattern of MDC fluorescence, consistent with autophagosomes, is identified in treated but not control cells.

In this study, we found that resveratrol inhibits the proliferation and survival of five malignant ovarian carcinoma cell lines. Cell cycle analysis demonstrated that resveratrol induces cell cycle arrest with the following features: after 12 h of exposure, removing the drug does not reverse growth arrest; moreover, cell cycle arrest is concentration dependent such that lower concentrations induce arrest at the G_2-M transition with accumulation in the S-phase, whereas concentrations >50 μM cause accumulation of cells in G_0-G_1 (see Fig. 2). These results are consistent with other reports showing that the antiproliferative effects of resveratrol are variably linked to either S-phase arrest, such as in MCF7 and HL60 cells, or G_1 arrest in A431 epidermoid carcinoma cells (25, 51).

We observed dose-dependent cytotoxic effects of resveratrol against each of the ovarian cell lines. Cell death, determined by plasma membrane permeability changes, is maximal by 48 h of treatment. We fully characterized cell death in A2780 cells, which, among the cell lines tested, was the most sensitive to resveratrol. Interestingly, although the apoptosome complex forms as an early response to treatment, cell death also occurs in the presence of apoptotic inhibitors, indicating that resveratrol can activate nonapoptotic cell death. Resveratrol induced cell death is independent of caspase function or Bcl-2/Bcl-x_L expression, cellular DNA is not degraded, and the resulting cellular morphology is not typical of apoptosis. Rather, our results point to autophagocytosis (autophagy or type II programmed cell death) as the main mode of death.

The involvement of an autophagocytic mechanism explains why particular cells that are resistant to cisplatin and other proapoptotic drugs, for example Bcl-2- and Bcl-x_L-expressing A2780 clones, or ES-2 cells, are sensitive to resveratrol. Resistance to apoptosis is a significant problem in ovarian cancer treatment and in some cases is understood to result from tumor cell defects in apoptosome function or from the expression of high levels of Bcl-2 or other antiapoptotic genes. Consequently, agents such as resveratrol, which are capable of killing by an alternative, nonapoptotic death mechanism, have considerable appeal as experimental agents against this disease. Along these lines, it is interesting to note that a previous study with human leukemia U937 cells found that high-level Bcl-2 attenuated resveratrol-induced cell death (apoptosis; Ref. 52). Therefore, resveratrol's ability to trigger autophagocytic death and overcome failed apoptosis may depend on the particular cell type involved.

Autophagy is a physiological mechanism that involves the sequestration of cytoplasm and intracellular organelles into membrane vacuoles called autophagosomes and results in their eventual enzymatic degradation (36). In response to appropriate stimulation, depolarized mitochondria are known to move into autophagic vacuoles. Thus, mitochondrial dysfunction may be a point of overlap between apoptotic and autophagocytic processes (53). The fusion of the edges of the membrane sac forms a closed double-membrane structure, the so-called autophagosome. Finally, the autophagosome fuses with a lysosome to become the autolysosome. Within autolysosomes, lysosomal hydrolases degrade the sequestered cellular constituents.

A2780 cells treated with resveratrol demonstrate an ultrastructural appearance consistent with the formation of autophagosomes. Furthermore, resveratrol-treated cells stain with MDC, a specific marker for autophagic vacuoles (37). Confirmatory experiments were performed using 3-MA to block autophagocytosis (54). In our experiments, however, 3-MA alone was toxic at appropriate concentrations, precluding an attempt to determine its capacity to block resveratrol killing (data not shown).

Adding autophagy to the list of resveratrol's bioactivities has implications for cancer treatment and chemoprevention. We have shown that although resveratrol-induced cell death can trigger formation of the apoptosome and apoptosis, an alternative pathway of cell death, autophagocytosis is also activated (see model, Fig. 8). Gene products that regulate autophagy also function as tumor suppressor genes, supporting the argument that this process is involved in the elimination of cancer cells by triggering a nonapoptotic cell death program (53, 55). One particularly interesting example is Beclin 1, a Bcl-2-interacting coiled-coil protein. Beclin 1 promotes autophagy when overexpressed in MCF-7 cells, and beclin-1 is monoallelically deleted in 40–75% of sporadic breast and ovarian cancers (56). Furthermore, treatment of MCF-7 cells with tamoxifen, a widely used preventive and therapeutic agent for breast cancer, has been shown to induce autophagocytosis (57). Experimentation is planned to determine whether resveratrol-induced death depends on Beclin-1 or other specific gene products thus far implicated in human cell autophagocytosis.

One practical question with resveratrol is whether serum or tissue concentrations can be attained that reach the levels at which growth arrest and autophagy are observed *in vitro*. In humans, there is an insufficient understanding of the pharmacokinetics and bioactivity of resveratrol and its metabolites to know this with certainty. In rats receiving an oral dose of 2 mg/kg, peak concentrations of 2.6 μM were achieved. Resveratrol is extensively glucuronidated and sulfated in the liver and is converted by the P450 isoenzyme CYP1B1 to piceatannol, which has antitumor cell properties of its own (58). For example, when resveratrol was given to mice as a 60-mg/kg oral dose, serum levels of resveratrol-glucuronide and -sulfate exceeded 100 and 300 μM, respectively (59). Although the bioactivity of these conjugated metabolites has not been tested, their hydrophilic properties make it unlikely that either will be accessible to intracellular targets. Nevertheless, β-glucuronidase activity is present at high levels in the extracellular space of certain bulky tumors, and enzymes with sulfatase activity are expressed at particularly high levels in gynecological tumors, including ovarian cancers (60, 61). These enzymes may be capable of converting the resveratrol metabolites back to *trans*-resveratrol, providing tumor-selective bioactivation and a sufficient concentration of active drug to induce autophagy.

Therapeutic utility of anticancer drugs is largely governed by selectivity against diseased tissue and tolerability of side effects relative to overall benefit. In animal models, resveratrol's toxicity is minimal, and even actively proliferating tissues are not adversely affected (*e.g.*, bone marrow, gastrointestinal tract). The maximum tolerated dose of resveratrol in mice is 4,000 mg/kg/day for 28 days.[4] Indeed, the minimal toxicity in animals has allowed resveratrol to enter human studies sponsored by the National Cancer Institute aimed at cancer prevention using healthy volunteers. We are beginning studies to determine whether resveratrol is active against ovarian cancer xenografts in mice to assess the degree to which the compound selectively targets tumor cells relative to normal tissues and to show whether autophagy is observed *in vivo*.

In summary, we have identified a novel activity for resveratrol in ovarian cancer cells, the ability to induce autophagocytosis. This response and its ability to growth arrest these cells hint at the possibility that this agent may be useful as an adjuvant therapy to treat ovarian tumors. Although resveratrol treatment engages certain components of the apoptotic machinery, important inhibitors of apoptosis, such as Bcl-2, do not limit its cytotoxic effects on these cells. These findings pave the way for additional experiments to consider the molecular basis for this response, a potential role for beclin-1, and studies to determine whether adequate concentrations of bioactive resveratrol can be attained to treat ovarian tumors.

[4] Dr. Tristan Booth, Royalmount Pharma, personal communication.

ACKNOWLEDGMENTS

We thank Drs. Kathleen Cho, Gabriel Nuñez, Michael Clarke, and Tristan Booth for helpful discussions.

REFERENCES

1. Jemal, A., Thomas, A., Murray, T., and Thun, M. Cancer statistics, 2002. CA Cancer. J. Clin., *52:* 23–47, 2002.
2. Kalil, N. G., and McGuire, W. P. Chemotherapy for advanced epithelial ovarian carcinoma. Best Pract. Res. Clin. Obstet. Gynaecol., *16:* 553–571, 2002.
3. Vikhanskaya, F., and Broggini, M. Genetic alterations in ovarian cancer cells that might account for sensitivity to chemotherapy in patients. Int. Rev. Cytol., *219:* 157–198, 2002.
4. Gadducci, A., Cosio, S., Muraca, S., and Genazzani, A. R. Molecular mechanisms of apoptosis and chemosensitivity to platinum and paclitaxel in ovarian cancer: biological data and clinical implications. Eur. J. Gynaecol. Oncol., *23:* 390–396, 2002.
5. Coukos, G., and Rubin, S. C. Chemotherapy resistance in ovarian cancer: new molecular perspectives. Obstet. Gynecol., *91:* 783–792, 1998.
6. Bhat, K. P., and Pezzuto, J. M. Cancer chemopreventive activity of resveratrol. Ann. NY Acad. Sci., *957:* 210–229, 2002.
7. Tessitore, L., Davit, A., Sarotto, I., and Caderni, G. Resveratrol depresses the growth of colorectal aberrant crypt foci by affecting bax and p21(CIP) expression. Carcinogenesis (Lond.), *21:* 1619–1622, 2000.
8. Carbo, N., Costelli, P., Baccino, F. M., Lopez-Soriano, F. J., and Argiles, J. M. Resveratrol, a natural product present in wine, decreases tumour growth in a rat tumour model. Biochem. Biophys. Res. Commun., *254:* 739–743, 1999.
9. Mgbonyebi, O. P., Russo, J., and Russo, I. H. Antiproliferative effect of synthetic resveratrol on human breast epithelial cells. Int. J. Oncol., *12:* 865–869, 1998.
10. Ragione, F. D., Cucciolla, V., Borriello, A., Pietra, V. D., Racioppi, L., Soldati, G., Manna, C., Galletti, P., and Zappia, V. Resveratrol arrests the cell division cycle at S/G2 phase transition. Biochem. Biophys. Res. Commun., *250:* 53–58, 1998.
11. Hsieh, T. C., Burfeind, P., Laud, K., Backer, J. M., Traganos, F., Darzynkiewicz, Z., and Wu, J. M. Cell cycle effects and control of gene expression by resveratrol in human breast carcinoma cell lines with different metastatic potentials. Int. J. Oncol., *15:* 245–252, 1999.
12. Hsieh, T. C., Juan, G., Darzynkiewicz, Z., and Wu, J. M. Resveratrol increases nitric oxide synthase, induces accumulation of p53 and p21(WAF1/CIP1), and suppresses cultured bovine pulmonary artery endothelial cell proliferation by perturbing progression through S and G_2. Cancer Res., *59:* 2596–2601, 1999.
13. Hsieh, T. C., and Wu, J. M. Differential effects on growth, cell cycle arrest, and induction of apoptosis by resveratrol in human prostate cancer cell lines. Exp. Cell Res., *249:* 109–115, 1999.
14. Banerjee, S., Bueso-Ramos, C., and Aggarwal, B. B. Suppression of 7,12-dimethylbenz(*a*)anthracene-induced mammary carcinogenesis in rats by resveratrol: role of nuclear factor-κB, cyclooxygenase 2, and matrix metalloprotease 9. Cancer Res., *62:* 4945–4954, 2002.
15. Bhat, K. P., Lantvit, D., Christov, K., Mehta, R. G., Moon, R. C., and Pezzuto, J. M. Estrogenic and antiestrogenic properties of resveratrol in mammary tumor models. Cancer Res., *61:* 7456–7463, 2001.
16. Bhat, K. P., and Pezzuto, J. M. Resveratrol exhibits cytostatic and antiestrogenic properties with human endometrial adenocarcinoma (Ishikawa) cells. Cancer Res., *61:* 6137–6144, 2001.
17. Bhat, K. P. L., Kosmeder, J. W., II, and Pezzuto, J. M. Biological effects of resveratrol. Antioxid. Redox. Signal, *3:* 1041–1064, 2001.
18. Bernhard, D., Tinhofer, I., Tonko, M., Hubl, H., Ausserlechner, M. J., Greil, R., Kofler, R., and Csordas, A. Resveratrol causes arrest in the S-phase prior to Fas-independent apoptosis in CEM-C7H2 acute leukemia cells. Cell Death Differ., *7:* 834–842, 2000.
19. Fukuhara, K., and Miyata, N. Resveratrol as a new type of DNA-cleaving agent. Bioorg. Med. Chem. Lett., *8:* 3187–3192, 1998.
20. Liu, J. R., Opipari, A. W., Tan, L., Jiang, Y., Zhang, Y., Tang, H., and Nunez, G. Dysfunctional apoptosome activation in ovarian cancer: implications for chemoresistance. Cancer Res., *62:* 924–931, 2002.
21. Liu, R., Page, C., Beidler, D. R., Wicha, M. S., and Nunez, G. Overexpression of Bcl-x_L promotes chemotherapy resistance of mammary tumors in a syngeneic mouse model. Am. J. Pathol., *155:* 1861–1867, 1999.
22. Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., and Riccardi, C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Methods, *139:* 271–279, 1991.
23. Kerr, J. F., and Harmon, B. V. Definition and incidence of apoptosis: an historical perspective. *In:* L. D. Tomei and F. O. Cope (eds.), Apoptosis: The Molecular Basis of Cell Death, pp. 5–29. Plainview, NY: Cold Spring Harbor Laboratory Press, 1991.
24. Pozo-Guisado, E., Alvarez-Barrientos, A., Mulero-Navarro, S., Santiago-Josefat, B., and Fernandez-Salguero, P. M. The antiproliferative activity of resveratrol results in apoptosis in MCF-7 but not in MDA-MB-231 human breast cancer cells: cell-specific alteration of the cell cycle. Biochem. Pharmacol., *64:* 1375–1386, 2002.
25. Ahmad, N., Adhami, V. M., Afaq, F., Feyes, D. K., and Mukhtar, H. Resveratrol causes WAF-1/p21-mediated G_1-phase arrest of cell cycle and induction of apoptosis in human epidermoid carcinoma A431 cells. Clin. Cancer Res., *7:* 1466–1473, 2001.
26. Tinhofer, I., Bernhard, D., Senfter, M., Anether, G., Loeffler, M., Kroemer, G., Kofler, R., Csordas, A., and Greil, R. Resveratrol, a tumor-suppressive compound from grapes, induces apoptosis via a novel mitochondrial pathway controlled by Bcl-2. FASEB J., *15:* 1613–1615, 2001.
27. Cain, K., Bratton, S. B., Langlais, C., Walker, G., Brown, D. G., Sun, X-M., and Cohen, G. M. Apaf-1 oligomerizes into biologically active ~700-kDa and inactive ~1.4-MDa apoptosome complexes. J. Biol. Chem., *275:* 6067–6070, 2000.
28. Rodriguez, J., and Lazebnik, Y. Caspase-9 and APAF-1 form an active holoenzyme. Genes Dev., *13:* 3179–3184, 1999.
29. 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.
30. Hu, Y., Benedict, M. A., 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.
31. Knaapen, M. W., Davies, M. J., De Bie, M., Haven, A. J., Martinet, W., and Kockx, M. M. Apoptotic *versus* autophagic cell death in heart failure. Cardiovasc. Res., *51:* 304–312, 2001.
32. Kanoh, M., Takemura, G., Misao, J., Hayakawa, Y., Aoyama, T., Nishigaki, K., Noda, T., Fujiwara, T., Fukuda, K., Minatoguchi, S., and Fujiwara, H. Significance of myocytes with positive DNA *in situ* nick end-labeling (TUNEL) in hearts with dilated cardiomyopathy: not apoptosis but DNA repair. Circulation, *99:* 2757–2764, 1999.
33. Watanabe, M., Hitomi, M., van der Wee, K., Rothenberg, F., Fisher, S. A., Zucker, R., Svoboda, K. K., Goldsmith, E. C., Heiskanen, K. M., and Nieminen, A. L. The pros and cons of apoptosis assays for use in the study of cells, tissues, and organs. Microsc. Microanal., *8:* 375–391, 2002.
34. Kinch, G., Hoffman, K. L., Rodrigues, E. M., Zee, M. C., and Weeks, J. C. Steroid-triggered programmed cell death of a motoneuron is autophagic and involves structural changes in mitochondria. J. Comp. Neurol., *457:* 384–403, 2003.
35. von Bultzingslowen, I., Jontell, M., Hurst, P., Nannmark, U., and Kardos, T. 5-Fluorouracil induces autophagic degeneration in rat oral keratinocytes. Oral Oncol., *37:* 537–544, 2001.
36. Reggiori, F., and Klionsky, D. J. Autophagy in the eukaryotic cell. Eukaryot. Cell, *1:* 11–21, 2002.
37. Biederbick, A., Kern, H. F., and Elsasser, H. P. Monodansylcadaverine (MDC) is a specific *in vivo* marker for autophagic vacuoles. Eur. J. Cell Biol., *66:* 3–14, 1995.
38. Eskelinen, E. L., Prescott, A. R., Cooper, J., Brachmann, S. M., Wang, L., Tang, X., Backer, J. M., and Lucocq, J. M. Inhibition of autophagy in mitotic animal cells. Traffic, *3:* 878–893, 2002.
39. Fremont, L. Biological effects of resveratrol. Life Sci., *66:* 663–673, 2000.
40. Jang, M., Cai, L., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W., Fong, H. H., Farnsworth, N. R., Kinghorn, A. D., Mehta, R. G., Moon, R. C., and Pezzuto, J. M. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science (Wash. DC), *275:* 218–220, 1997.
41. Li, Z. G., Hong, T., Shimada, Y., Komoto, I., Kawabe, A., Ding, Y., Kaganoi, J., Hashimoto, Y., and Imamura, M. Suppression of *N*-nitrosomethylbenzylamine (NMBA)-induced esophageal tumorigenesis in F344 rats by resveratrol. Carcinogenesis (Lond.), *23:* 1531–1536, 2002.
42. Schneider, Y., Duranton, B., Gosse, F., Schleiffer, R., Seiler, N., and Raul, F. Resveratrol inhibits intestinal tumorigenesis and modulates host-defense-related gene expression in an animal model of human familial adenomatous polyposis. Nutr. Cancer, *39:* 102–107, 2001.
43. Roemer, K., and Mahyar-Roemer, M. The basis for the chemopreventive action of resveratrol. Drugs Today (Barc.), *38:* 571–580, 2002.
44. Manna, S. K., Mukhopadhyay, A., and Aggarwal, B. B. Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-κB, activator protein-1, and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation. J. Immunol., *164:* 6509–6519, 2000.
45. Holmes-McNary, M., and Baldwin, A. S., Jr. Chemopreventive properties of *trans*-resveratrol are associated with inhibition of activation of the IκB kinase. Cancer Res., *60:* 3477–3483, 2000.
46. Subbaramaiah, K., Chung, W. J., Michaluart, P., Telang, N., Tanabe, T., Inoue, H., Jang, M., Pezzuto, J. M., and Dannenberg, A. J. Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. J. Biol. Chem., *273:* 21875–21882, 1998.
47. Delmas, D., Passilly-Degrace, P., Jannin, B., Malki, M. C., and Latruffe, N. Resveratrol, a chemopreventive agent, disrupts the cell cycle control of human SW480 colorectal tumor cells. Int. J. Mol. Med., *10:* 193–199, 2002.
48. Kuwajerwala, N., Cifuentes, E., Gautam, S., Menon, M., Barrack, E. R., and Reddy, G. P. Resveratrol induces prostate cancer cell entry into S phase and inhibits DNA synthesis. Cancer Res., *62:* 2488–2492, 2002.
49. Asensi, M., Medina, I., Ortega, A., Carretero, J., Bano, M. C., Obrador, E., and Estrela, J. M. Inhibition of cancer growth by resveratrol is related to its low bioavailability. Free Radical Biol. Med., *33:* 387–398, 2002.
50. Brakenhielm, E., Cao, R., and Cao, Y. Suppression of angiogenesis, tumor growth, and wound healing by resveratrol, a natural compound in red wine and grapes. FASEB J., *15:* 1798–1800, 2001.
51. Joe, A. K., Liu, H., Suzui, M., Vural, M. E., Xiao, D., and Weinstein, I. B. Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. Clin. Cancer Res., *8:* 893–903, 2002.
52. Park, J. W., Choi, Y. J., Suh, S. I., Baek, W. K., Suh, M. H., Jin, I. N., Min, D. S., Woo, J. H., Chang, J. S., Passaniti, A., Lee, Y. H., and Kwon, T. K. Bcl-2 overexpression attenuates resveratrol-induced apoptosis in U937 cells by inhibition of caspase-3 activity. Carcinogenesis (Lond.), *22:* 1633–1639, 2001.
53. Ogier-Denis, E., and Codogno, P. Autophagy: a barrier or an adaptive response to cancer. Biochim. Biophys. Acta, *1603:* 113–128, 2003.

54. Seglen, P. O., and Gordon, P. B. 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. Proc. Natl. Acad. Sci. USA, *79:* 1889–1892, 1982.
55. Arico, S., Petiot, A., Bauvy, C., Dubbelhuis, P. F., Meijer, A. J., Codogno, P., and Ogier-Denis, E. The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. J. Biol. Chem., *276:* 35243–35246, 2001.
56. Liang, X. H., Yu, J., Brown, K., and Levine, B. Beclin 1 contains a leucine-rich nuclear export signal that is required for its autophagy and tumor suppressor function. Cancer Res., *61:* 3443–3449, 2001.
57. Bursch, W., Ellinger, A., Gerner, C., Frohwein, U., and Schulte-Hermann, R. Programmed cell death (PCD). Apoptosis, autophagic PCD, or others? Ann. NY Acad. Sci., *926:* 1–12, 2000.
58. Potter, G. A., Patterson, L. H., Wanogho, E., Perry, P. J., Butler, P. C., Ijaz, T., Ruparelia, K. C., Lamb, J. H., Farmer, P. B., Stanley, L. A., and Burke, M. D. The cancer preventative agent resveratrol is converted to the anticancer agent piceatannol by the cytochrome P450 enzyme CYP1B1. Br. J. Cancer, *86:* 774–778, 2002.
59. Yu, C., Shin, Y. G., Chow, A., Li, Y., Kosmeder, J. W., Lee, Y. S., Hirschelman, W. H., Pezzuto, J. M., Mehta, R. G., and van Breemen, R. B. Human, rat, and mouse metabolism of resveratrol. Pharm. Res., *19:* 1907–1914, 2002.
60. Schumacher, U., Adam, E., Zangemeister-Wittke, U., and Gossrau, R. Histochemistry of therapeutically relevant enzymes in human tumours transplanted into severe combined immunodeficient (SCID) mice: nitric oxide synthase-associated diaphorase, β-D-glucuronidase and non-specific alkaline phosphatase. Acta Histochem., *98:* 381–387, 1996.
61. Okuda, T., Saito, H., Sekizawa, A., Shimizu, Y., Akamatsu, T., Kushima, M., Yanaihara, T., Okai, T., and Farina, A. Steroid sulfatase expression in ovarian clear cell adenocarcinoma: immunohistochemical study. Gynecol. Oncol., *82:* 427–434, 2001.