

Heterogeneous Role of Caspase-8 in Fenretinide-Induced Apoptosis in Epithelial Ovarian Carcinoma Cell Lines

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ABSTRACT

The mechanism of action of fenretinide, a synthetic retinoid currently undergoing testing as a chemopreventive and chemotherapeutic agent, is incompletely understood. In the present study, fenretinide caused apoptotic changes, including DNA fragmentation and cleavage of caspase substrates, in six low-passage ovarian cancer cell lines. However, the caspase activation pathway used by this agent varied. Transient transfection of cDNA-encoding cytokine response modifier A (CrmA), a caspase-8 inhibitor, diminished fenretinide-induced death in OV177 cells. Likewise, IETD(OMe)-fluoromethylketone (fmk) inhibited fenretinide-induced apoptosis by >80% in OV177 or OV266 cells and by ~50% in OV17, OV167, or OV207 cells. Further analysis demonstrated that inhibition of Fas ligand, tumor necrosis factor- α , or TRAIL signaling with blocking reagents did not affect fenretinide-induced apoptosis, raising the

possibility that fenretinide activates caspase-8 in a death receptor-independent manner. In contrast, CrmA transfection or IETD(OMe)-fmk treatment did not inhibit fenretinide-induced apoptosis in OV202 cells. These divergent behaviors did not correlate with increased levels of procaspase-10, which is relatively resistant to CrmA and IETD(OMe)-fmk, nor with the expression of procaspase-8 and -9, apoptotic protease activating factor-1, or cellular FLICE-like inhibitory protein. Similarly, fenretinide treatment increased ceramide levels equally in cells that do (OV177) and do not (OV202) rely on caspase-8 to initiate apoptosis. These results indicate that synthetic retinoids can use caspase-8 as an initiating caspase, but they also indicate unexpected heterogeneity in caspase activation pathways among closely related cell lines.

The synthetic retinoid *N*-(4-hydroxyphenyl)retinamide (fenretinide) is currently undergoing widespread clinical testing as a potential antineoplastic agent directed against a variety of solid tumors (Swerdlow et al., 2001). Initially identified as a potential chemopreventive agent, fenretinide has possible application for both chemoprevention and chemotherapy because it induces apoptosis in cell lines derived from a variety of malignancies. The biochemical pathways leading to fenretinide-induced apoptosis are complex, reportedly involving both retinoid receptor-dependent (Fanjul et al., 1996) and -independent (Delia et al., 1993; Sheikh et al.,

1995; Chiantore et al., 1999; Clifford et al., 1999) effects, reactive oxygen species (Hail and Lotan, 2001), and ceramide production (Maurer et al., 1999). Whereas preliminary clinical data suggested the specific use of fenretinide as an agent effective against ovarian cancer (De Palo et al., 1995, 2002), characterization of the effects of fenretinide in ovarian carcinoma cells remains incomplete.

In addition to their primary effects at proximal targets, a variety of anticancer drugs trigger apoptosis, a morphologically and biochemically distinct cell-death process that reflects the activity of a unique family of cysteine proteases called caspases (Herr and Debatin, 2001; Mow et al., 2001). There are two well-established pathways for activating these enzymes (Herr and Debatin, 2001; Mow et al., 2001). One pathway is triggered by mitochondrial release of cytochrome

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ABBREVIATIONS: FADD, Fas-associated death domain polypeptide; RA, retinoic acid; ATRA, all-*trans*-retinoic acid; Apaf-1, apoptotic protease activating factor-1; CrmA, cytokine response modifier A; c-FLIP, cellular FLICE-like inhibitory protein; GFP, green fluorescent protein; fmk, fluoromethylketone; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; PKC δ , protein kinase C δ ; SFM, serum-free medium; TLC, thin-layer chromatography; TNF- α , tumor necrosis factor- α ; topo, topoisomerase; TRAIL, TNF- α -related apoptosis inducing ligand; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; XIAP, X chromosome-linked inhibitor of apoptosis; EGFP, enhanced green fluorescent protein.

c, which binds the cytosolic protein Apaf-1, thereby facilitating the binding and activation of procaspase-9. Once activated, caspase-9 can activate caspase-3, which is responsible for the majority of caspase-mediated apoptotic cleavages within cells. The second pathway starts with the ligation of death receptors, which recruit adapter molecules such as FADD that bind and activate procaspase-8 and -10. In some lymphohematopoietic cells ("type I cells"), caspase-8 and/or -10 can also activate caspase-3. In other cells ("type II cells"), the limited amounts of caspase-8 generated by death-receptor signaling cleave the proapoptotic Bcl-2 family member Bid to liberate a fragment that facilitates mitochondrial cytochrome c release and caspase-9-mediated caspase-3 activation (Herr and Debatin, 2001; Mow et al., 2001).

A number of studies using Fas-resistant cell lines or cells from FADD^{-/-} mice have demonstrated that many antineoplastic agents do not require an intact death receptor pathway to trigger apoptosis (Herr and Debatin, 2001; Mow et al., 2001). More recently, however, caspase-8 has been implicated in the action of a small but growing number of agents. In colon cancer cells, for example, 5-fluorouracil-induced thymidine depletion is accompanied by p53-mediated Fas ligand up-regulation, which seems to be critical for subsequent cell death (Mow et al., 2001). Similarly, TRAIL up-regulation seems to play a role in the antileukemic action of ATRA in acute promyelocytic leukemia tissue culture cells and clinical samples (Altucci et al., 2001). Additional studies have implicated caspase-8 as the initiating caspase during cell death induced by the topoisomerase poisons etoposide (Micheau et al., 1999) and camptothecin (Shao et al., 2001), as well as the chemopreventive agent 2-cyano-3,12-dioxolean-1,9-dien-28-oic acid (Ito et al., 2000).

The apoptotic pathway triggered by fenretinide remains unsettled. Studies in various cell types have led to the suggestion that fenretinide might trigger apoptosis by stabilizing caspase-3 (DiPietrantonio et al., 2000) or activating caspase-8, possibly in a Fas-independent manner (You et al., 2001). Alternatively, the induction of apoptosis has been attributed to mitochondrial dysfunction (Hail and Lotan, 2001) leading to cytochrome c release (Suzuki et al., 1999) and caspase-9 activation (Ulukaya et al., 2003). Because the death receptor and mitochondrial pathways are inhibited by different apoptotic regulators (Herr and Debatin, 2001; Mow et al., 2001; Meng et al., 2002), elucidation of the pathway used by fenretinide has potential implications for mechanisms of fenretinide resistance.

In the present study, we investigated the antiproliferative effects of fenretinide in a series of previously characterized low-passage ovarian carcinoma cell lines (Conover et al., 1998). These studies were designed to examine the identity of the caspases that are activated by fenretinide in these cells and, upon demonstration that both caspase-8 and -9 are activated, to evaluate the respective roles of these initiator caspases in fenretinide-induced apoptosis. Surprisingly, results of this analysis suggest that fenretinide uses procaspase-8 to varying degrees as the initiator caspase even within this one tumor cell type. These results not only have important implications for current understanding of the mechanism of action of fenretinide but also suggest previously unrecognized variability in caspase activation pathways after drug treatment.

Materials and Methods

Materials

Fenretinide was a gift of the R. W. Johnson Research Institute (Spring House, NJ). ATRA, 9-*cis*-RA, and paclitaxel were purchased from Sigma Chemical (St. Louis, MO). Stock solutions in absolute ethanol (fenretinide) or 1:1 ethanol/dimethyl sulfoxide (ATRA, 9-*cis*-RA) were protected from light and stored at -20°C. Additional reagents were purchased from the following suppliers: IETD(OMe)-fmk was from Enzyme Systems Products (Livermore, CA); anti-Fas monoclonal antibodies CH-11 and ZB-4 were from Kamiya Biomedical (Thousand Oaks, CA); agonistic recombinant human soluble TRAIL, Enhancer Product, and antagonistic fusion protein TRAIL-R2:Fc were from Alexis (San Diego, CA); and J1D9 neutralizing anti-TNF- α antibody was from Ancell (Bayport, MN).

Cell Culture

Epithelial ovarian carcinoma cell lines derived from primary tumors of previously untreated women with ovarian cancer were cultured as described previously (Conover et al., 1998). Cells were grown to 50 to 80% confluence before treatment with death-inducing stimuli. In some experiments, cells were switched to SFM (phenol red-free Dulbecco's minimal essential medium containing 0.1% (w/v) bovine serum albumin, 2 mM L-glutamine, and antibiotics) before drug treatment. A549 human lung cancer cells and Jurkat T cell leukemia cells were cultured in RPMI 1640 medium containing 5 or 15% heat-inactivated fetal bovine serum, respectively, as well as 100 units/ml penicillin G, 100 μ g/ml streptomycin, and 2 mM glutamine. The Jurkat variants I2.1 (lacking FADD) and I9.2 (lacking procaspase-8) from American Type Culture Collection (Manassas, VA) were cultured according to instructions from the supplier.

Proliferation Assays

[³H]Thymidine incorporation was analyzed as described previously (Conover et al., 1998). Triplicate determinations for each condition are represented as a percentage of [³H]thymidine incorporation in solvent-treated cells.

To assess cell mass by MTT dye reduction, cells were plated in 96-well plates and allowed to attach overnight. Retinoids were added for 48 h in either complete medium or SFM, after which MTT was added to a final concentration of 0.5 mg/ml. After a 1-h incubation, supernatants were aspirated, the wells were washed with phosphate-buffered saline, and the precipitated formazan was solubilized with 100 μ l of dimethyl sulfoxide. Absorbance at 595 nm with a reference blank at 630 nm was read using an automated plate reader. Quadruplicate wells were analyzed for each condition.

Assays for Apoptosis

Morphological Analysis. Subconfluent cells were treated with fenretinide or solvent for 72 h. Nonadherent and adherent cells were pooled, fixed in Trump's fixative, and stained with 1 μ g/ml Hoechst 33258 in 50% glycerol containing 0.1 M Tris-HCl, pH 7.4 at 21°C. For each treatment, 500 to 800 cells were scored for apoptotic changes (peripheral chromatin condensation or nuclear fragmentation) under epi-illumination using an Axioplan microscope (Carl Zeiss GmbH, Jena, Germany) equipped with a numerical aperture 1.40, 63 \times objective, a 365 nm excitation filter, and a 420 nm emission filter.

Flow Cytometry. Adherent and nonadherent cells were pooled, washed in Dulbecco's calcium-free, magnesium-free phosphate-buffered saline, fixed in 50% ethanol, treated with 100 μ g/ml RNase A, and stained with 100 μ g/ml propidium iodide for flow cytometric analysis using a FACscan Plus cytometer (BD Biosciences, San Jose, CA). Debris was excluded by gating on forward and side scatter.

TUNEL. OV177 cells were treated with fenretinide in SFM for 24, 48, and 72 h. At the end of the incubation, adherent and nonadherent cells were pooled, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and processed using the In Situ Cell Death

Detection kit (Roche Diagnostics, Indianapolis, IN), which labels DNA strand breaks with fluorescein dUTP. Flow cytometry was performed as described above.

Immunoblotting

OV177 cells were treated with fenretinide or diluent in SFM at 37°C for 48 or 72 h. Adherent and nonadherent cells were collected separately, subjected to electrophoresis, and transferred to nitrocellulose as described previously (Kottke et al., 2002). Blots were probed with mouse monoclonal antibodies against PARP, lamin A, procaspase-9, or topo I provided by Guy Poirier (Laval University, St. Foy, Quebec, Canada), Frank McKeon (Harvard University, Cambridge, MA), Yuri Lazebnik (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), and Y.-C. Cheng (Yale University, New Haven, CT), respectively. Alternatively, blots were probed with reagents that recognize gelsolin, procaspase-3, procaspase-7, or procaspase-8 (BD Biosciences Pharmingen, San Diego, CA), procaspase-6 (Upstate Biotechnology, Lake Placid, NY), procaspase-10 (Medical and Biological Laboratories, Nagoya, Japan), lamin B₁, the nucleolar protein B23, or epitopes generated upon activation of caspase-3 and caspase-9 (Kottke et al., 2002). After reaction with horseradish peroxidase-coupled secondary antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD), blots were visualized using enhanced chemiluminescence reagents (Amersham Biosciences Inc., Piscataway, NJ).

Transcriptional Profiling

After OV177 cells were treated with solvent or 5 μ M fenretinide for 36 h, adherent and nonadherent cells were pooled for RNA preparation using the RNeasy Total RNA Isolation kit (QIAGEN, Valencia, CA). The 36-h time point was chosen because ~90% of the cells remained adherent despite the prolonged exposure and impending apoptosis. RNA quality assessment, probe labeling, and hybridization to the Affymetrix U95A Human Genome Array Chip were performed by the Mayo Clinic Cancer Center Microarray Shared Resource.

Ceramide Production

OV202 and OV177 cells were seeded in 6-well plates and were allowed to reach 50% confluence. Radiolabeling, lipid extraction, and analysis of [³H]ceramide by TLC were performed as described previously (Wang et al., 2001). Briefly, 2 μ Ci/well of [9,10-³H (N)]palmitic acid (Specific activity, 45 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) was added concurrently with solvent or 10 μ M fenretinide. Cells were harvested 24, 36, or 48 h after treatment. Adherent and nonadherent cells were pooled and washed twice in phosphate-buffered saline before biphasic extraction of lipids (Maurer et al., 1999; Wang et al., 2001). Solvent was dried under nitrogen, and lipids were stored at -20°C. After TLC resolution, iodine-visualized ceramide spots were scraped and analyzed for ³H-labeling by liquid scintillation counting. Zonal profiling was performed on representative samples to verify that the label was incorporated into ceramide. Results from triplicate wells for each experimental condition were expressed as mean \pm S.D. of [³H]ceramide cpm per 100,000 total lipid cpm.

Transfection

Using a BTX 820 square-wave electroporator for two 5-ms pulses of 320 (OV177) or 340 (OV202) volts, 2 \times 10⁶ cells per curette in the buffer described by van den Hoff et al. (1992) were transfected with a total of 20 μ g of DNA consisting of pEGFP-N1 alone, a 5:1 ratio of plasmid-encoding CrmA in the sense or antisense orientation (kindly provided by Dr. Charles Young, Mayo Clinic, Rochester, MN) along with pEGFP-N1, or plasmid-encoding GFP-dn caspase-9 (Srinivasula et al., 1998). Electroporated cells were plated in complete medium without antibiotics and were allowed to adhere at 37°C for 20 to 24 h. Cells were changed to SFM and treated with diluent or 5 μ M

fenretinide for 72 h before processing for Hoechst staining as above. Under these conditions, 50 to 60% of the cells displayed GFP fluorescence in each experiment.

A549 and Jurkat cells were transfected with the same plasmids using a single 10-ms pulse of 240 V. GFP-expressing cells were isolated by flow cytometry before fenretinide treatment. After incubation for 24 h in the media described above, cells were treated with diluent or fenretinide, as indicated in the figure legends. Alternatively, Jurkat cells were treated for 24 h with 10 ng/ml CH-11 agonistic anti-Fas antibody.

Statistical Analysis

Data shown are representative of the indicated number of replicate experiments. Differences in paired groups were analyzed using a χ^2 pairwise analysis with *p* values indicated in the text.

Results

Fenretinide Decreases the Growth of Epithelial Ovarian Carcinoma Cell Lines. In the present study, six low-passage epithelial ovarian carcinoma lines (Conover et al., 1998) were used to study the apoptotic pathways activated by fenretinide. Before embarking on these studies, we first verified that the response of these cell lines was similar to that reported previously for other cancer cell lines. For these experiments, cells were exposed to fenretinide continuously to mimic the prolonged exposure achieved clinically by long-term daily administration.

In initial experiments, incorporation of [³H]thymidine into DNA was measured. Fenretinide inhibited [³H]thymidine incorporation in a dose-dependent manner in all six lines, with mean IC₅₀ values ranging from 1 to 5 μ M (Fig. 1A). In contrast, ATRA and 9-*cis*-RA, natural retinoids used in treatment of other malignancies, did not appreciably inhibit DNA synthesis in any cell lines tested but instead increased [³H]thymidine incorporation in some of the cell lines (Fig. 1B).

To determine whether the decreased thymidine incorporation reflected decreased viable cell mass, MTT reduction was examined in four of the cell lines. As illustrated for OV177 cells, fenretinide decreased the viable cell mass of all four cell lines in serum-containing medium (Fig. 1C) and SFM (Fig. 1D). MTT reduction decreased to <10% of control values, indicating that fenretinide treatment was not merely inhibiting cell proliferation, but it was also inducing death. In contrast, ATRA and 9-*cis*-RA did not decrease MTT reduction (Fig. 1, C and D).

Fenretinide Induces Apoptosis. Several different approaches were used to confirm that fenretinide was inducing apoptosis. Hoechst 33258 staining demonstrated chromatin condensation and nuclear fragmentation, morphological features of apoptosis, in fenretinide-treated OV177 cultures but not control cultures (Fig. 2, B and A inset, respectively). Similar changes were seen after OV17, OV167, OV202, OV207, and OV266 were treated with fenretinide (K.R. Kalli, unpublished observations). Consistent with these results, a subpopulation of cells with extractable DNA ("subdiploid cells"), a sign of endonucleosomal cleavage, was seen when parallel cultures were fixed in ethanol, stained with propidium iodide, and subjected to flow cytometry (Fig. 2B). As indicated in Fig. 2C, the percentage of cells with extractable DNA increased after 48 and 72 h of fenretinide treatment but not after ATRA. Likewise, TUNEL staining using fluorescein

isothiocyanate-labeled dUTP showed labeling of fenretinide-treated cells (Fig. 2D), providing additional evidence of endonucleolytic DNA degradation. In short, fenretinide induced classic apoptotic changes, including chromatin condensation, nuclear fragmentation, and DNA degradation.

Fenretinide Induces Caspase Activation. To determine whether fenretinide-induced cell death was accompanied by caspase activation in these cells, cleavage of caspase zymogens and caspase substrates was examined by immunoblotting. After OV177 cells were treated with fenretinide for 48 or 72 h, whole-cell lysates were separately prepared from adherent and nonadherent cells. Aliquots containing equal amounts of total cellular protein were subjected to SDS-polyacrylamide gel electrophoresis and probed with reagents that recognize caspase substrates (Fig. 3A) or caspase zymogens and active caspases (Fig. 3B).

Examination of various caspase substrates revealed that nonadherent cells harvested after fenretinide treatment contained decreased levels of intact PARP, gelsolin, lamin B₁, and lamin A. Fragments corresponding in size to previously reported caspase cleavage products of PARP, topo I, PKC δ , and lamin B₁ (Kottke et al., 2002) were also detected in the nonadherent cells using these reagents (Fig. 3A, arrows). As was the case in other epithelial cell lines undergoing drug-induced apoptosis [e.g., breast cancer cells (Kottke et al.,

2002)], adherent cells contained only the intact forms of these polypeptides (Fig. 3A). This finding of cleaved substrates only in nonadherent cells reflects the observation that apoptotic cells detach from tissue culture substrata.

Immunoblotting of the same whole-cell lysates showed that the precursors for caspase-3, -6, -7, -8, -9, and -10 were all decreased in nonadherent cells after fenretinide treatment (Fig. 3B). Fragments corresponding to the molecular weights of active subunits of caspase-3, -8, and -9 were seen in the nonadherent populations only (Fig. 3B, arrows). Constant levels of B23, a nucleolar protein that does not undergo apoptosis-associated proteolysis, demonstrated equivalent loading of all lanes.

Fenretinide Induces Apoptosis in OV177 Cells via a Pathway Sensitive to Both Dominant-Negative Caspase-9 and CrmA. In an effort to determine whether altered expression of components of one of the two well-established caspase activation pathways (see Introduction) might contribute to fenretinide-induced apoptosis, mRNA prepared from OV177 cells treated with solvent or 5 μ M fenretinide was used to probe the U95A Human Genome array (Affymetrix, Santa Clara, CA). In this experiment, stress-regulated messages such as those encoding osteopontin were up-regulated more than 20-fold. In contrast, mRNA-encoding death receptors, death ligands, and members of the

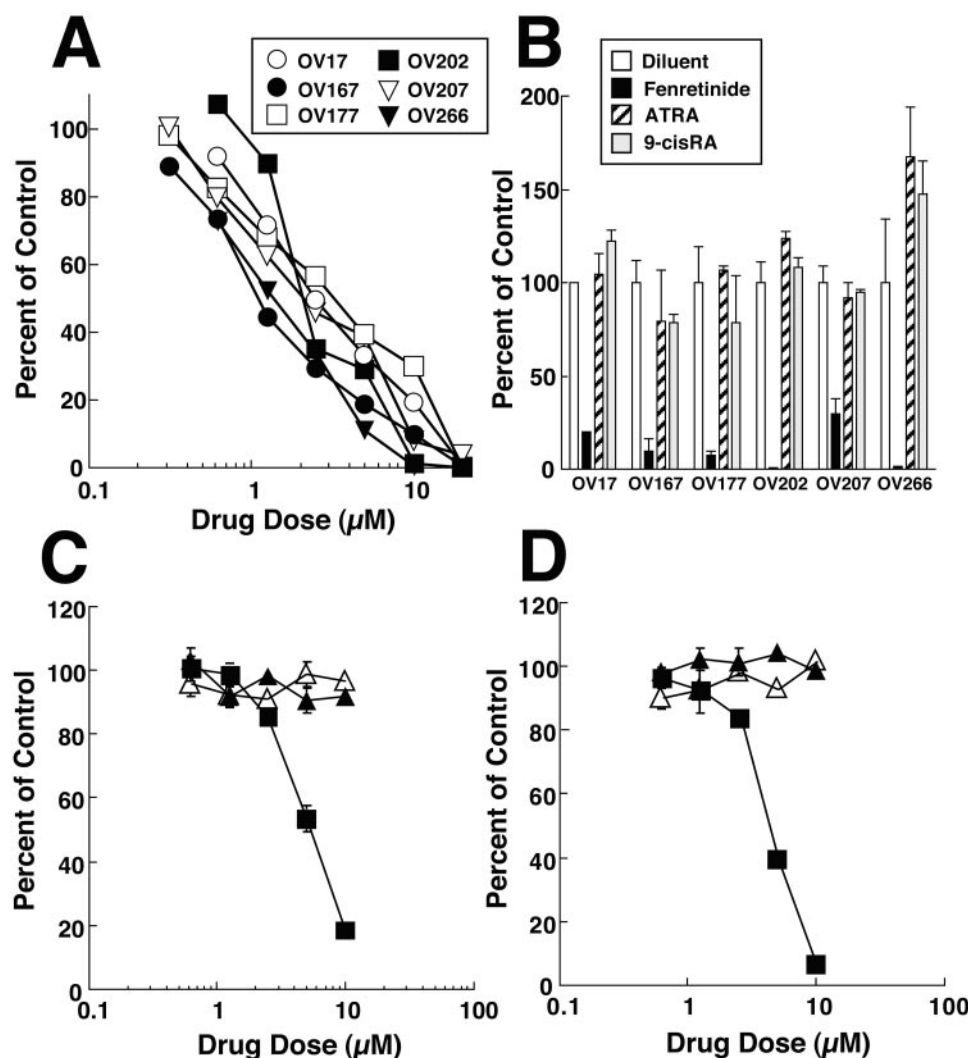


Fig. 1. Antiproliferative effects of fenretinide. A, after the indicated cells were treated with fenretinide for 48 h, incorporation of [3 H]thymidine into DNA was analyzed as described under *Materials and Methods*. Symbols represent means of two to seven experiments. Error bars indicating pooled variances (generally <10%) were omitted for clarity. B, [3 H]thymidine incorporation in cells treated for 48 h with diluent (□), 10 μ M fenretinide (■), 10 μ M ATRA (▨) or 9-cisRA (▩) in the same experiments shown in A. C and D, OV177 cells were treated with the indicated concentrations of fenretinide (■), ATRA (△), or 9-cisRA (▲) in serum-containing (C) or serum-free medium (D) for 48 h and analyzed for MTT reduction as described under *Materials and Methods*.

Bcl-2/Bax families changed less than 3-fold after fenretinide treatment and did not correlate with the observed cytotoxicity (data not shown). Because of this result, alternative approaches were used to investigate the mechanism of fenretinide-induced apoptosis.

Because fenretinide induces apoptosis very slowly in the overall cell population (Fig. 2C), whereas the execution phase of apoptosis has been shown previously to occur rapidly once initiated in individual cells, time-course experiments were believed to be unlikely to distinguish between caspase-8 and -9 as the initiating caspase (Fig. 3B). Instead, expression constructs and small-molecule caspase inhibitors that target defined steps of the two pathways were applied in subsequent experiments. Previous studies demonstrated that processes initiated by caspase-8 can be blocked by caspase-8 inhibitors, whereas processes that are initiated by caspase-9 cannot be blocked by these agents even if caspase-9 induces downstream proteolytic activation of procaspase-8 (Slee et al., 1999; Zhou and Salvesen, 2000). In the present study, OV177 cells were transfected with cDNA-encoding CrmA, a serpin that inhibits caspase-1 and -8 relatively selectively (Zhou and Salvesen, 2000), or dominant-negative procaspase-9 fused to GFP, which inhibits procaspase-9 activation (Srinivasula et al., 1998). Plasmid encoding CrmA in the anti-sense orientation served as a control. In these experiments, 50 to 60% of cells were successfully transfected as monitored by expression of GFP (cotransfected with CrmA or fused to procaspase-9). Because attempts to purify the trans-

fected cells by flow cytometry resulted in unacceptable levels of apoptosis under control conditions, studies were performed on the bulk transfected population.

In these studies, OV177 cells were exposed to fenretinide or diluent for 72 h beginning 24 h after transfection. At the completion of the incubation, adherent and nonadherent cells were pooled, fixed, stained with Hoechst 33258, and scored for apoptotic morphologic changes (Fig. 4A). A similar percentage of cells transfected with GFP or the CrmA antisense construct underwent apoptosis after fenretinide treatment. Transfection of dominant-negative caspase-9 or the CrmA sense construct reduced the percentage of cells that were apoptotic by ~50%, the theoretical maximal effect for experiments with 50% transfection efficiency (determined by fluorescence microscopy for GFP expression). The ability of both dominant-negative caspase-9 and CrmA to inhibit fenretinide-induced apoptosis is consistent with a process that is initiated by procaspase-8 in a type II cell (Scaffidi et al., 1998). In contrast, paclitaxel-induced apoptosis, which has previously been reported to involve the mitochondrial pathway (Perkins et al., 1998; Kottke et al., 2002), was decreased by transfection with dominant-negative caspase-9 but not by CrmA (Fig. 4B), providing assurance that the protective effect of CrmA in fenretinide-treated OV177 cells does not result from a nonspecific antiapoptotic function of the construct.

Fas, TNFR1, and TRAIL Receptor-Independent Fenretinide-Induced Caspase Activation in OV177 Cells.

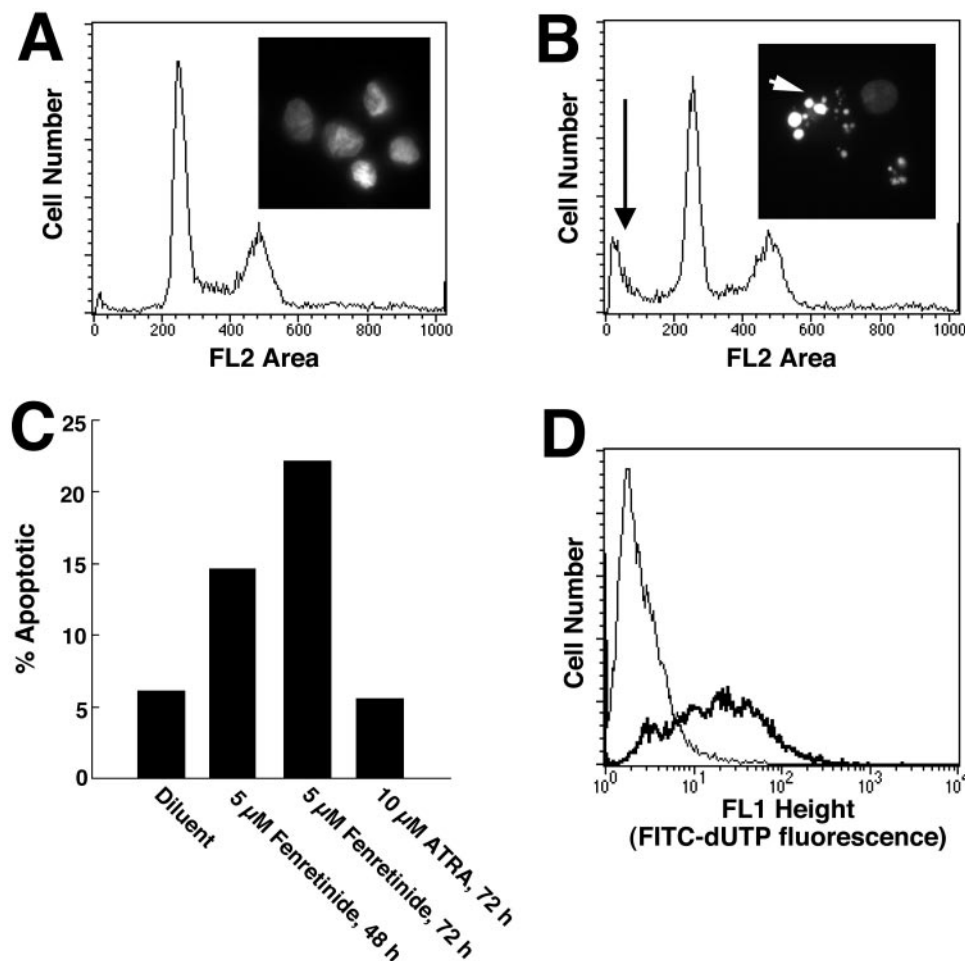


Fig. 2. Fenretinide induces apoptotic changes in OV177 cells. A and B, OV177 cells were treated for 72 h with diluent (A) or 5 μ M fenretinide (B), after which adherent and nonadherent cells were pooled, fixed, stained with propidium iodide, and subjected to flow cytometry. Cells were gated by forward and side scatter to exclude debris. Black arrow, the apoptotic population. Insets, cells were fixed, stained with Hoechst 33258, and examined under UV illumination. The white arrow indicates one of several cells with apoptotic morphology. C, OV177 cells treated with diluent, 48 h of fenretinide, 72 h of fenretinide, or 72 h of ATRA were analyzed for extractable DNA as described under *Materials and Methods*. D, TUNEL analysis. Cells were treated with diluent (thin line) or fenretinide (thick line) for 72 h, fixed, treated with terminal deoxynucleotidyl transferase in the presence of fluorescein isothiocyanate-labeled dUTP, and subjected to flow cytometry.

To distinguish between receptor-dependent and receptor-independent caspase activation, we initially attempted to assess the effect of dominant-negative FADD, which blocks death receptor-mediated caspase activation (Herr and Debatin, 2001; Mow et al., 2001; Thorburn et al., 2003a), on fenretinide-induced apoptosis. Unfortunately, truncated FADD was toxic to the OV177 cells, as has been reported previously in prostate cells (Thorburn et al., 2003a). As an alternative, blocking constructs were used to assess the role

of various death-receptor pathways in fenretinide-induced apoptosis.

To investigate whether Fas/Fas ligand interactions were involved, monoclonal anti-Fas antibodies that have agonistic (CH-11) or antagonistic (ZB-4) effects on Fas-sensitive cells were used. The antagonistic antibody ZB-4 had no effect on the induction of apoptosis by fenretinide during a 48- or 72-h incubation (Fig. 5A). Additionally, agonistic CH-11 antibody induced only limited apoptosis in OV177 cells even though the same antibody efficiently induced apoptosis in a number of lymphoid cell lines (Meng et al., 2002). The inability of blocking Fas antibodies to alter the cytotoxicity of fenretinide, coupled with the relative resistance of OV177 cells to Fas-mediated apoptosis, argues against the possibility that signaling through Fas is responsible for fenretinide-induced apoptosis.

Reagents that activate or block TRAIL receptors also failed to influence fenretinide-mediated apoptosis. When OV177 cells were treated for 48 and 72 h with fenretinide in the presence of DR5 (TRAIL-R2):Fc, a soluble chimera that binds TRAIL and prevents its interaction with TRAIL receptors, fenretinide-induced apoptosis was not inhibited (Fig. 5B). Control experiments demonstrated the ability of this construct to inhibit TRAIL-induced apoptosis in OV177 cells (Fig. 5B, inset) and Jurkat cells (X. Meng and S. H. Kaufmann, unpublished observations), confirming the efficacy of the receptor blockade. Conversely, cross-linked TRAIL had only a weak proapoptotic effect on OV177 cells even though this reagent induced apoptosis very effectively in Jurkat cells in our laboratory (Meng et al., 2002; data not shown). Collectively, the inability of the blocking reagent to alter fenretinide-induced apoptosis and the relative insensitivity of OV177 cells to TRAIL-induced apoptosis argue against the possibility that signaling through TRAIL receptors is responsible for fenretinide-induced apoptosis.

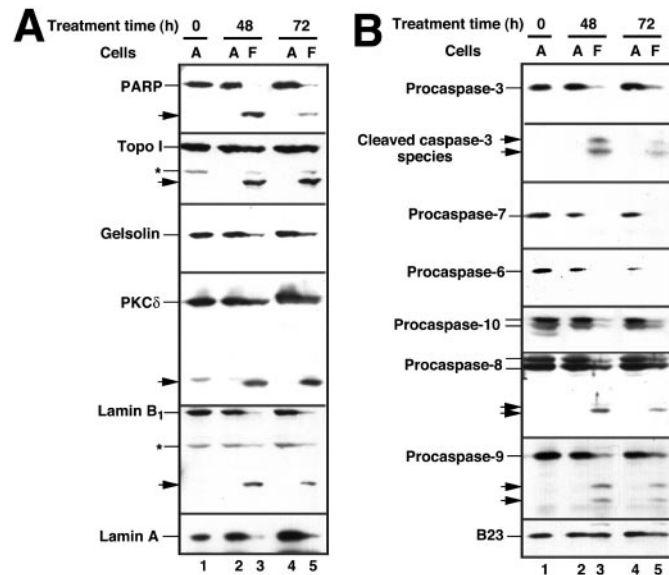


Fig. 3. The effect of fenretinide on selected caspase substrates and procaspases. A, OV177 cells that became nonadherent (F) or remained adherent (A) after treatment with 5 μ M fenretinide for 48 or 72 h were harvested and subjected to immunoblotting with reagents that recognize the caspase substrates PARP, topo I, gelsolin, PKC δ , lamin B₁, or lamin A. B, the identical lysates were probed using reagents that recognize procaspase-3, -6, -7, -8, -9, and -10. All lanes were loaded with 50 μ g of protein, and the nucleolar protein B23 is shown as a loading control. Bars, intact proteins; arrows, fenretinide-induced breakdown products or active caspases; *, nonspecific cross-reactive bands.

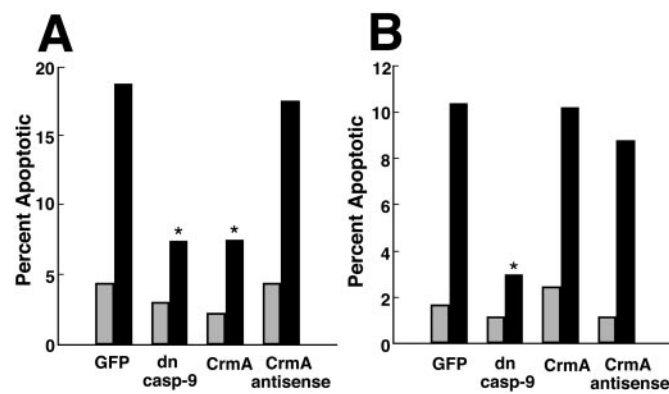


Fig. 4. Fenretinide triggers apoptosis in OV177 cells through a CrmA-sensitive pathway. A, OV177 cells were transfected with constructs encoding GFP, CrmA in the sense or antisense orientation, or dominant-negative caspase-9. After treatment with diluent (□) or 5 μ M fenretinide (■), cells were fixed, stained with Hoechst 33258, and examined for apoptotic morphological changes. B, transfected cells were treated for 24 h with 100 nM paclitaxel, washed, transferred to drug-free medium, and incubated for an additional 72 h. Adherent and nonadherent cells were pooled, stained with Hoechst 33258, and examined for apoptotic morphological changes. Results are representative of three separate experiments. *, $p < 0.0001$ relative to fenretinide treatment of GFP-transfected cells.

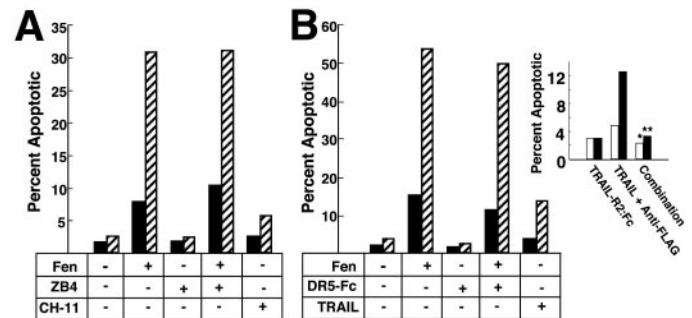


Fig. 5. Fenretinide-induced apoptosis is unaffected by anti-Fas antibodies or TRAIL receptor antagonists. A, OV177 cells were treated for 48 h (■) or 72 h (▨) with the agonistic antibody CH-11, fenretinide alone, or fenretinide in combination with the antagonistic anti-Fas antibody ZB-4. After fixation and staining with Hoechst 33258, cells were examined for apoptotic morphological changes. B, reagents blocking the interaction with TRAIL do not influence fenretinide-mediated killing. OV177 cells were treated for 48 h (■) or 72 h (▨) with the antagonist recombinant human (rh)TRAIL-R2:Fc (DR5-Fc) in the presence or absence of fenretinide. Alternatively, cells were treated with agonistic rhTRAIL aggregated with anti-FLAG antibodies. At the completion of the treatments, cells were fixed, stained with Hoechst 33258, and examined. Results in each panel are representative of at least three independent experiments. Inset, control showing OV177 cells treated with the rhTRAIL-R2:Fc-soluble (blocking) TRAIL receptor alone, TRAIL and anti-FLAG, or the combination for 72 h (□) or 96 h (■) before processing for Hoechst staining as above. *, $p = 0.023$ and **, $p < 0.0001$ relative to cells treated with TRAIL and anti-FLAG for the same length of time.

Similarly, J1D9, a monoclonal antibody that neutralizes soluble TNF- α , was ineffective in preventing fenretinide-induced apoptosis in OV177 cells, but did prevent TNF- α -induced apoptosis in Jurkat cells (K. R. Kalli, unpublished observations). TNF- α alone was weakly effective in killing OV177 cells, with levels of apoptosis after 72 h that were similar to the levels induced by TRAIL or Fas agonists (data not shown). Collectively, these data indicate that neither

Fas, TRAIL receptors, nor TNFR1 is responsible for fenretinide-mediated activation of procaspase-8.

Heterogeneity in the Use of Caspase-8 to Initiate Fenretinide-Induced Apoptosis. To examine independently the potential roles of procaspase-8 and -9, cells were treated with IETD(OMe)-fmk, which inhibits caspase-8 in preference to caspase-9 (Garcia-Calvo et al., 1998). Treatment with 10 μ M IETD(OMe)-fmk inhibited fenretinide-in-

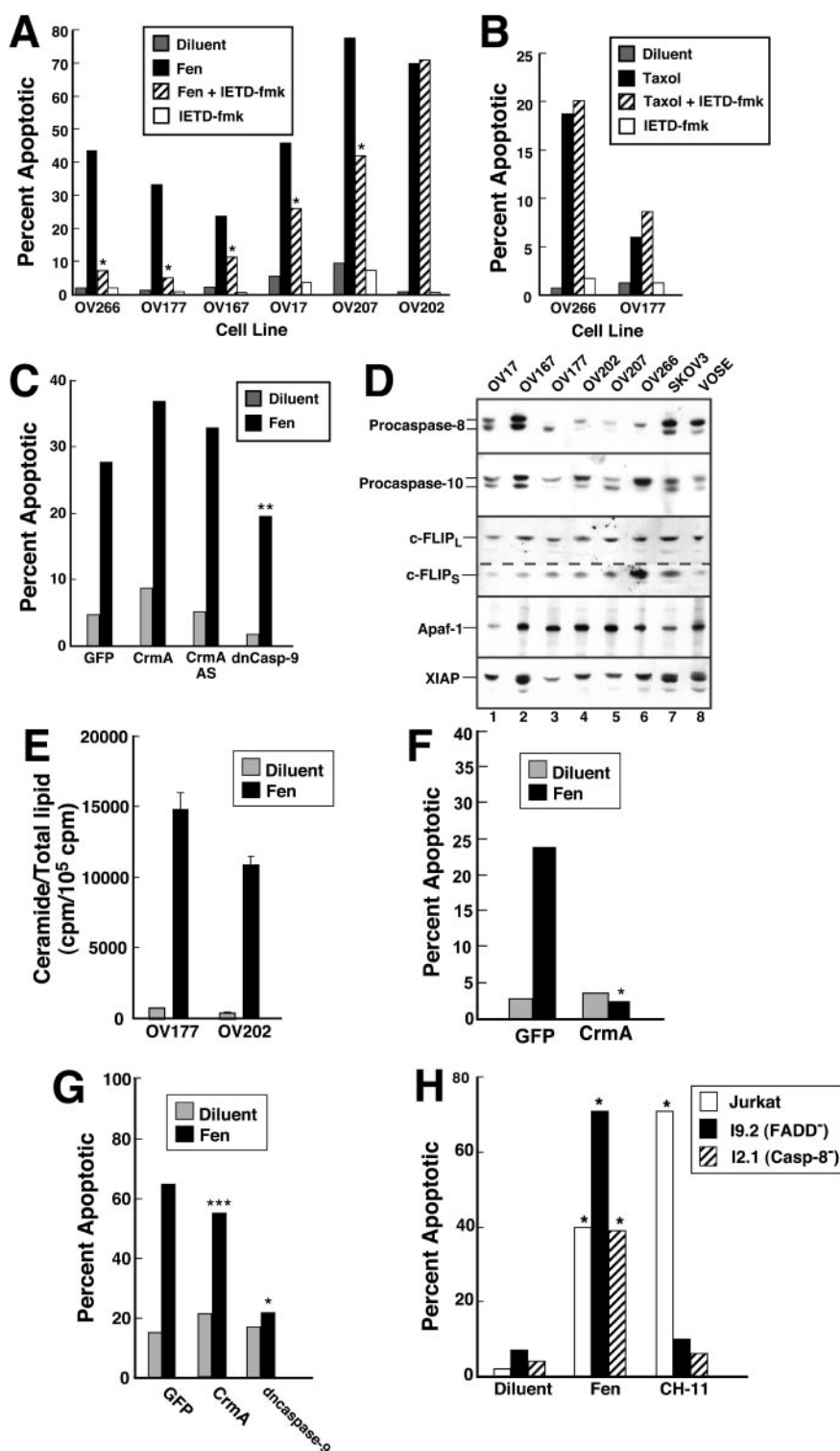


Fig. 6. Heterogeneous ability of IETD(OMe)-fmk and CrmA to inhibit fenretinide-induced apoptosis. A, cell lines were grown to 60 to 80% confluence and treated with fenretinide (Fen) in the presence and absence of 10 μ M IETD(OMe)-fmk. Seventy-two hours after treatment, cells were fixed, stained with Hoechst 33258, and examined for apoptotic morphological changes as described above. Results are representative of three to five experiments in each cell line. *, $p < 0.0001$ relative to fenretinide alone. B, cells were treated for 24 h with 100 nM paclitaxel, washed, transferred to drug-free medium, and incubated for an additional 72 h in the absence or presence of 10 μ M IETD(OMe)-fmk, after which cells were fixed and stained as described above for microscopic examination of apoptotic changes. C, OV202 cells were transfected with constructs encoding GFP, CrmA in the sense or antisense orientation, or dominant-negative caspase 9. After treatment with diluent (□) or 5 μ M fenretinide (■), cells were fixed, stained with Hoechst 33258, and examined for apoptotic morphological changes. **, $p = 0.027$ relative to fenretinide-treated GFP-transfected cells. D, lysates containing 50 μ g of protein from the indicated cell lines were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with reagents that recognize procaspase-8, procaspase-10, c-FLIP, Apaf-1, or XIAP. E, OV177 and OV202 cells were treated with vehicle or fenretinide in the presence of [³H]palmitic acid. After 36 h, lipids were extracted and analyzed for [³H]ceramide by TLC. F and G, effect of CrmA on fenretinide-induced apoptosis in A549 (F) and Jurkat cells (G). Cells were transfected with EGFP-N1 alone or in a 4:1 ratio of plasmids encoding CrmA and EGFP. Twenty-four hours after transfection, EGFP-expressing cells were isolated by flow cytometry and then treated with 5 μ M (F) or 1.25 μ M fenretinide (G) versus diluent for 48 (F) or 24 h (G). *, $p < 0.0001$ and ***, $p = 0.014$ relative to fenretinide in GFP-transfected cells. H, effect of caspase-8 or FADD deficiency on apoptosis in Jurkat cells. Parental Jurkat cells or derivatives lacking caspase-8 (12.1) or FADD (19.2) were incubated for 24 h with diluent, fenretinide, or 10 ng/ml CH-11 agonistic anti-Fas antibody. Immunoblotting (data not shown) confirmed the absence of caspase-8 and FADD in the respective cell lines. *, $p < 0.0001$ versus diluent.

duced apoptosis by >80% in OV177 cells (Fig. 6A). Similar results were observed in OV266 cells. Paclitaxel (Taxol)-induced apoptosis was not affected by IETD(OMe)-fmk in either cell line (Fig. 6B), providing evidence for selectivity of the inhibition. Further analysis, however, indicated that the effect of IETD(OMe)-fmk on fenretinide-induced apoptosis varied among the remaining ovarian cancer cell lines (Fig. 6A), with OV17, OV167, and OV207 showing 40 to 50% inhibition in the presence of 10 μ M IETD(OMe)-fmk and OV202 showing no inhibition in any of five experiments analyzed at varying levels of cell death.

In view of these results, the ability of CrmA to influence fenretinide-induced apoptosis was examined in OV202 cells. Once again, cells were transfected under conditions in which 50 to 60% of cells exhibited GFP fluorescence; however, transfected cells could not be isolated because of the toxicity of the sorting process. Consistent with the results observed with IETD(OMe)-fmk, transfection with CrmA failed to inhibit fenretinide-induced apoptosis in OV202 cells (Fig. 6C).

Previous studies have demonstrated that CrmA inhibits caspase-10 less effectively than it does caspase-8 (Zhou and Salvesen, 2000). Additional experiments have demonstrated that caspase-10 is also less sensitive to IETD-fmk (T. J. Kottke and S. H. Kaufmann, unpublished observations). To determine whether the varied behavior of ovarian cancer cells might correlate with expression of caspase-10 rather than caspase-8, whole-cell lysates were probed with reagents that recognize each zymogen. Results of this analysis (Fig. 6D) failed to show any correlation between the inhibition of fenretinide-induced apoptosis by IETD(OMe)-fmk and caspase-10 expression. Likewise, there was no correlation between IETD(OMe)-fmk sensitivity and levels of c-FLIP, an endogenous regulator of caspase-8 and caspase-10 activation (Krueger et al., 2001). Additional blotting also failed to identify a relationship between the expression of procaspase-9, Apaf-1, or XIAP and IETD(OMe)-fmk sensitivity (Fig. 6D and data not shown). Finally, the ability of fenretinide to up-regulate total ceramide did not distinguish between cell lines that used caspase-8 to initiate fenretinide-induced apoptosis and those that did not. In agreement with previous studies (Maurer et al., 1999; Wang et al., 2001; Prinetti et al., 2003), ceramide levels increased 20- to 30-fold after a 36-h fenretinide treatment (Fig. 6E). Similar changes were observed at 24 and 48 h as well (data not shown). These changes, however, occurred in both OV177 cells and OV202 cells, indicating that ceramide up-regulation is unlikely to be a factor in the differential requirement for caspase-8 to trigger fenretinide-induced apoptosis in OV177 cells.

In further experiments, the effect of CrmA on fenretinide-induced apoptosis was assessed in diverse cell lines. Fenretinide-induced apoptosis in A549 non-small-cell lung cancer cells was almost completely inhibited by CrmA (Fig. 6F). In contrast, CrmA had little effect on fenretinide-induced apoptosis in Jurkat T cell leukemia cells (Fig. 6G) even though dominant-negative caspase-9 completely abolished this process. Consistent with these results, Jurkat cell variants lacking FADD or caspase-8 (I9.2 and I2.1 cells, respectively) were completely resistant to CH-11 agonistic anti-Fas antibody but remained sensitive to fenretinide (Fig. 6H). Collectively, the observations in Fig. 6, F through H, confirm the observation that fenretinide induces apoptosis by a CrmA-inhibitable pathway in some cell lines (Fig. 6F) but

also verify that the predominance of this pathway varies from cell line to cell line (Fig. 6, G and H, versus F).

Discussion

Previous studies have not only identified fenretinide as a potential chemopreventive and chemotherapeutic agent but also documented its ability to induce apoptosis in a variety of neoplastic cell lines. The present study extends these earlier results by demonstrating heterogeneity in initiator caspase usage after fenretinide treatment of a series of ovarian cancer cell lines. In most lines tested, fenretinide triggers apoptosis through a process that requires, at least to some extent, involvement of caspase-8. In other cell lines, however, fenretinide induces apoptosis independent of caspase-8. These observations have potentially important implications for the future development of fenretinide.

The present study examined a series of low-passage ovarian cancer cell lines with genetic similarities to the tumors from which they were derived (Conover et al., 1998). Before studying the manner in which fenretinide triggered apoptosis, we first determined that the responses of these lines to fenretinide were similar to those reported previously for other cells. The six ovarian cancer lines were profoundly inhibited by 0.5 to 10 μ M fenretinide (Fig. 1), a result that agrees with previously reported IC₅₀ values in the 0.7 to 2.7 μ M range for a number of ovarian cancer cell lines (Um et al., 2001). Fenretinide exhibited these antiproliferative effects, whereas ATRA and 9-*cis*-RA did not (Fig. 1), in agreement with previous suggestions that the antiproliferative effects of fenretinide might be independent of RAR and RXR receptors (Delia et al., 1993; Sheikh et al., 1995; Chiantore et al., 1999; Clifford et al., 1999). Finally, ceramide levels were elevated by fenretinide (Fig. 6E) before caspases were activated, as has been reported previously in other cell lines (Maurer et al., 1999; Wang et al., 2001; Prinetti et al., 2003).

Likewise, there was nothing particularly distinctive about the effector phase of apoptosis in these cells. Fenretinide induced typical apoptotic changes, including detachment from the tissue-culture substratum, nuclear fragmentation, and DNA cleavage (Fig. 2). Further analysis demonstrated that substrates of caspase-3 (topo I, gelsolin, and PKC δ) and -6 (lamin A) were cleaved (Fig. 3A). Consistent with these observations caspase-3, -6, and -7 were activated (Fig. 3B). These results agree with previous descriptions of fenretinide-induced apoptosis in other model systems (Puduvalli et al., 1999; Suzuki et al., 1999; DiPietrantonio et al., 2000; Ulukaya et al., 2003).

Subsequent experiments focused on the caspases that initiate this process. Immunoblotting demonstrated that caspase-8, -9, and -10 are also cleaved in fenretinide-treated cells (Fig. 3B). Although the activation of caspase-8 and -10 occurs in conjunction with death-receptor-initiated apoptosis (Herr and Debatin, 2001; Mow et al., 2001), these caspases can also be cleaved downstream of caspase-3 when apoptosis is initiated by cytochrome *c* release (Slee et al., 1999; Kottke et al., 2002). Likewise, caspase-9 can be activated as a consequence of death-receptor ligation (in type II cells) or as a result of events that directly affect mitochondria (Scaffidi et al., 1998; Mow et al., 2001). Thus, the presence of cleaved caspase-8, -9 and -10 in fenretinide-treated OV177 does not indicate the pathway used by fenretinide to trigger apoptosis.

Further experiments revealed that CrmA inhibited fenretinide-induced apoptosis in OV177 cells (Fig. 4A). This result, coupled with earlier data showing that CrmA can inhibit caspase-8 but not caspase-3, -6, or -7 (Zhou and Salvesen, 2000), suggests that caspase-8 initiates caspase activation in fenretinide-treated OV177 cells. Indeed, if caspase-8 were merely activated downstream of caspase-9 and -3, CrmA transfection would have no effect, as is the case after treatment with DNA-damaging agents (Newton and Strasser, 2000) or paclitaxel (Fig. 4B). The observation that dominant-negative caspase-9 also diminishes fenretinide-induced apoptosis (Fig. 4A) would be consistent with the idea that OV177 cells, like many epithelial cell lines, are type II cells that activate caspase-9 downstream of caspase-8 (Scaffidi et al., 1998). The conclusion that caspase-8 is the initiating caspase for fenretinide-induced apoptosis in OV177 cells is further supported by the observation that IETD(OMe)-fmk, which contains the preferred peptide-recognition sequence of caspase-8, also inhibited fenretinide-induced apoptosis in five of the six cell lines (Fig. 6A), albeit to varying degrees.

Although the present results did not identify the basis for fenretinide-induced activation of caspase-8 in OV177 cells, they ruled out a number of potential mechanisms. The inhibition of signaling through Fas, TNFR1, and TRAIL receptors failed to inhibit fenretinide-induced apoptosis (Fig. 5). Moreover, OV177 cells were resistant to killing by agonistic anti-Fas antibodies, TNF- α , and TRAIL. Taken together, these observations suggest that signaling through the most widely studied death receptors is not critical for fenretinide-induced apoptosis in this cell line. Whereas signaling through another death receptor cannot be ruled out, it is more likely that fenretinide activates caspase-8 in a death-ligand-independent fashion, as was reported recently for a granulocyte/macrophage colony-stimulating factor/diphtheria toxin conjugate (Thorburn et al., 2003b).

This involvement of caspase-8 in fenretinide-induced cytotoxicity has several important implications. First, alterations in the caspase-8 pathway might contribute to fenretinide resistance. The high levels of c-FLIP observed in certain cancers can inhibit caspase-8 (Krueger et al., 2001). In addition, diminished caspase-8 expression has been observed in several neoplasms, including neuroblastoma and small-cell lung cancer (Mow et al., 2001). Cells with these alterations might be resistant to fenretinide-induced apoptosis. Second, the participation of caspase-8 might affect the efficacy of certain drug combinations. Recent studies have demonstrated that the cytotoxicity of TRAIL can be markedly enhanced by DNA-damaging agents (Wen et al., 2000). Because fenretinide also uses caspase-8 as an initiator caspase, similar synergism might be observed if fenretinide were combined with DNA-damaging agents. Previous studies demonstrating that fenretinide enhances the efficacy of cisplatin (Supino et al., 1996) are consistent with this hypothesis. Additional studies are needed to further explore the postulated mechanisms of resistance and predicted drug-drug interactions in preclinical models and clinical samples from patients receiving fenretinide.

Despite the involvement of caspase-8 in fenretinide-induced apoptosis in five of the ovarian cell lines (Fig. 6A), neither IETD(OMe)-fmk nor CrmA inhibited this process in OV202 cells (Fig. 6, A and C). The failure of these treatments

to affect fenretinide-induced apoptosis was not caused by over- or under-stimulation of the OV202 cells with fenretinide, for similar results were observed at drug concentrations that resulted in apoptosis of 10 to 70% of the cells (K.R. Kalli, unpublished observations). Although this result would be conveniently explained if levels of the CrmA-resistant protease caspase-10 or c-FLIP were particularly high in OV202 cells, or if levels of the caspase-9 modulator XIAP were particularly low, the present results did not support these explanations (Fig. 6D). Moreover, fenretinide caused similar increases in ceramide levels (Fig. 6E) in cells that require caspase-8 to initiate apoptosis (OV177) and cells that do not (OV202), indicating that changes in total ceramide content do not determine pathway usage. Additional studies demonstrated that CrmA almost completely inhibited fenretinide-induced apoptosis in A549 cells (Fig. 6F) but not Jurkat cells (Fig. 6G), confirming the heterogeneity among cell lines. Collectively, these observations not only demonstrate that a single agent can use different pathways in widely divergent cell types (e.g., A549 lung cancer cells versus Jurkat T cell leukemia cells) but also indicate for the first time that heterogeneity in the usage of apoptotic pathways can be observed in carcinoma cell lines with the same histological origin (epithelial ovarian cancer cell lines OV177 versus OV202). These results suggest that the interactions between anticancer drugs and the apoptotic machinery might be more complex and more variable than previously suspected.

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