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Progesterone Induces Apoptosis in TRAIL-Resistant Ovarian Cancer Cells by Circumventing c-FLIP$_L$ Overexpression

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Abstract Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) holds great potential as an anticancer drug, since it induces selective cell death in cancer cells but not in normal ones. However, cancer cells often acquire resistance to TRAIL, which hinders its clinical efficacy. We previously demonstrated that progesterone triggers apoptosis in human ovarian cancer (OCa) cells. In the present study, we evaluated the prospect of utilizing progestins in combination with TRAIL to enhance cell death in TRAIL-sensitive (OVCA 420, OVCA 429, and OVCA 433) and -resistant (OVCA 432) OCa cell lines. TRAIL sensitivity (60–80% cell kill) bore no correlation with expression of the TRAIL receptors (DR4, DR5) or their decoys (DcR1 and DcR2), but was associated with activation of caspase-8 and -3, and downregulation of the long isoform of FLICE-like inhibitory protein (c-FLIP$_L$), an anti-apoptosis mediator. Small interfering RNA-mediated knockdown of c-FLIP$_L$ expression restored TRAIL sensitivity in OVCA 432 cells. Induction of c-FLIP$_L$ overexpression increased TRAIL resistance in TRAIL-sensitive lines. Thus, persistent high level of c-FLIP$_L$ expression likely mediates TRAIL resistance in OCa cells. Treatment of OCa cells with progesterone enhanced TRAIL-induced cell death (>85%), but only in TRAIL-sensitive cell lines. Combined treatment with two progestins was superior to single progestin treatment, with progesterone plus medroxyprogesterone acetate (MPA) achieving over 85% cell kill in both TRAIL-sensitive and -resistant OCa cell lines. Significantly, unlike TRAIL, progestin-induced cell death did not involve c-FLIP$_L$ downregulation. Hence, combined progestin regimens, with or without TRAIL, may serve as an effective therapy for OCa by circumventing the anti-apoptotic action of c-FLIP$_L$. J. Cell. Biochem. 102: 442–452, 2007. © 2007 Wiley-Liss, Inc.

Key words: ovarian surface epithelial cells; medroxyprogesterone acetate; norethisterone acetate; hormone replacement therapy

Ovarian epithelial cancer (OCa), derived from human ovarian surface epithelial cells (HOSE), represents approximately 90% of all ovarian malignancies in women [Abedini et al., 2004], and accounts for the highest death rate among carcinomas of the female reproductive tract. Standard therapies include surgery, combination chemotherapy with platinum plus a taxane, and radiotherapy. Although OCa tends to respond to chemotherapy, recurrence is frequent [Cancer Facts & Figures, 2007]. Thus, current research is focusing on the design of more efficient and less toxic agents to eradicate OCa cells.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor (TNF) superfamily, triggers apoptosis in a variety of malignant cells without marked toxicity on normal cells [Kelley and Ashkenazi, 2004]. Apoptosis is initiated by binding of TRAIL to the death domains (DD) of death receptors DR4 and/or DR5, which are widely expressed in most human cells. TRAIL can also interact with two non-functional decoy receptors, DcR1 and DcR2, without eliciting cell death [Kelley and Ashkenazi, 2004]. In OCa cells, TRAIL has been shown to induce apoptosis alone or in combination with chemotherapeutic agents [Vignati et al., 2002; Lane et al., 2004; Tomek et al.,

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2004; Liu et al., 2006]. However, resistance of OCa cells to TRAIL-induced apoptosis is a frequent event [Horak et al., 2005; Lane et al., 2006], which hinders the efficacy of TRAIL as a therapy for OCa.

Epidemiological data support a protective action of progestins against OCa [Ho, 2003]. Exposure of the human ovarian surface epithelium to high levels of progesterone during pregnancy appears to confer protection in subsequent years [Yen, 1994], and increased parity is associated with a lower risk for OCa [Adami et al., 1994; Salazar-Martinez et al., 1999]. Interestingly, twin-pregnancies afford better protection than singleton-pregnancies, probably due to greater levels of circulating progesterone achieved in the former condition [Batra et al., 1978]. A lower OCa incidence has been reported in oral contraceptive users taking formulations with high versus low progestin content [Rodriguez et al., 2002]. In animal models, the synthetic progestin medroxyprogesterone acetate (MPA) decreased the frequency of spontaneous development of OCa in the laying hen [Barnes et al., 2002], while OCa incidence was significantly lower in monkeys treated with levonorgestrel than in those untreated or treated with ethinylestradiol [Rodriguez et al., 1998].

We previously demonstrated that progesterone, at concentrations ranging from 10^{-8} to 10^{-6} M, exerts significant growth inhibition in normal and malignant HOSE cells [Syed et al., 2001] by stimulation of an extrinsic apoptosis signaling pathway involving upregulation of the Fas ligand and activation of caspase-8 [Syed and Ho, 2003]. These findings led us to hypothesize that combined treatment of OCa cells with TRAIL, a TNF superfamily ligand, and a synthetic progestin approved for clinical use could be an effective therapy for OCa. Synthetic progestins, such as MPA and norethisterone acetate (NETA) [Affandi, 2002; Stahlberg et al., 2004], have a half-life and bioavailability superior to those of the natural hormone [Schindler et al., 2003; Sitruk-Ware 2003], and are widely used as contraceptives and for hormone replacement therapy (HRT).

In the present study, we have compared the apoptotic-inducing capacity of TRAIL, progesterone, and the synthetic progestins MPA and NETA, individually or in combination, in OCa cells. The origin of TRAIL-resistance and a mechanism to override it have been proposed.

MATERIALS AND METHODS

Reagents

Cell growth medium contained MCDB105/Medium 199 (1:1) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), 1.5 g sodium bicarbonate, 1% L-Glutamine, and 1% penicillin/streptomycin (Gibco BRL, Carlsbad, CA). Progesterone, MPA, and NETA were from Sigma, and recombinant human TRAIL from R & D Systems (Minneapolis, MN). The caspase inhibitors z-DEDV-fmk and z-IETD-fmk were purchased from BD Biosciences (Clontech, Palo Alto, CA).

Cell Lines and Culture Conditions

The ovarian cancer cell lines OVCA 420, OVCA 429, OVCA 432, and OVCA 433 were established from patients with late-stage serous ovarian carcinomas [Bast et al., 1981]. The immortalized normal ovarian surface epithelial cell lines HOSE 12–12 and HOSE 642 [Tsao et al., 1995] were used as control. The phenotypes and culture conditions of these cell lines have been previously described [Syed et al., 2002, 2005a,b; Ho et al., 2003; Syed and Ho, 2003].

Treatment of Ovarian Cell Lines

Ovarian cell cultures at 80% confluence were harvested and seeded at a density of 2×10^5 cells in 25 cm^2 culture flasks containing cell growth medium. After 24 h, the medium was replaced with medium containing charcoal-stripped (CS)-FBS. To study the effect of progesterone, MPA, NETA, and/or recombinant human TRAIL, cell cultures were treated daily with different concentrations of progestins (10^{-9}–10^{-6} M) or TRAIL (0.1 or 1–330 ng/ml) for 3 days. To examine the possible synergism between different treatments, cells were cotreated with progesterone (10^{-6} M) and synthetic progestins (10^{-6} M) or TRAIL (33 ng/ml for OVCA 420 cells, 330 ng/ml for others) for 3 days.

Cell Viability Assay

One thousand cells per well were plated in 96 well-plates. Cytotoxicity was determined using the CellTiter96® AQueous One Solution cell proliferation assay (Promega, Madison, WI) according to the manufacturer's instructions. Cells were cultured for 3 days in the presence of progesterone, MPA, NETA, or TRAIL, alone or

in combination. On the fourth day, 20 μl of CellTiter 96® AQueous One Solution Reagent were added into each well of the 96-well-assay plate containing the samples in 100 μl of culture medium. Absorbance was measured at 490 nm using a microtiter plate reader. Absorbance of untreated cells was set as 100% viability, and absorbance of cell-free wells containing medium was set as zero.

RNA Isolation and Reverse Transcription-PCR Analysis

Total RNA was isolated using TRI-reagent (Sigma) according to protocols provided by the manufacturer. Hot-start PCR with AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, CT) was used for all amplification reactions. The thermocycler program for the human genes $FLIP_L$ (Forward: 5′-AATTCAAGGCTCAGAAGCGA-3′, Reverse: 5′-GGCAGAAACTCTGCTGTTCC-3′); $FLIP_S$ (Forward: 5′-ACCTTGTGGTTGAGTTGGAGAAAC-3′, Reverse: 5′-ACAATTTCCAAGAAT TTTCAGATCAG-3′); TRAIL receptor DR4 (Forward: 5′-GCCTGTAACCGGTGCACAGA-3′, Reverse: 5′-CATGGGAGGCAAGCAAACAA-3′); TRAIL receptor DR5 (Forward: 5′-CCTGCTGGATGCCTTGGA-3′, Reverse: 5′-CCAGAGCTCAACAAGTGGTCCT-3′); TRAIL decoy receptor DcR1 (Forward: 5′-TGGCCCCACAGCAACAGA-3′, Reverse 5′-CAGGGGTTACAGGCTCCAGTAT-3′); and TRAIL decoy receptor DcR2 (Forward: 5′-CTTACCTCAAAGGCATCTGCTCA-3′, Reverse: 5′-CATTGTCCTCCGCCCCA-3′) consisted of 32 cycles (for TRAIL receptors) and 35 cycles (for $FLIP_L$ and $FLIP_S$) of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, except for beta-actin (Forward: 5′-CCAACCGCGAGAAGATGAC-3′, Reverse: 5′-GGAAGGAAGGCTGGAAGAGT-3′), for which only 18 cycles were performed. Signal intensities of TRAIL receptors and FLIP amplicons were normalized to those of beta-actin to produce arbitrary units of relative abundance.

Western Blot

Cell lysates were prepared in RIPA buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% deoxycholate, 1.0% NP40, and 0.1% SDS] supplemented with a protease inhibitor mixture solution (Roche Molecular Biochemicals, Mannheim, Germany). After sonication, cell debris was pelleted by centrifugation, and protein concentration was determined with the BCA Protein Assay Reagent (Pierce, Rockford, IL). Equivalent amounts of proteins were resolved by 10% SDS–PAGE and transferred to nitrocellulose membranes. The blots were then incubated with a primary antibody to human c-FLIP (dilution 1:500) (Alexis Biochemicals, Lausanne, Switzerland), which recognizes both short and long spliced variants of c-FLIP, overnight at 4°C. A secondary polyclonal antibody to mouse IgG1, horseradish peroxidase-conjugated, (Alexis Biochemicals) was used. The Enhanced Chemiluminescence Detection System (Amersham Pharmacia Biotech, Piscataway, NJ), followed by autoradiography, was used for protein visualization.

Caspase Activity Assays

Cells were pretreated for 1 h with the caspase-3 inhibitor z-DEDV-fmk or the caspase-8 inhibitor z-IETD-fmk, before the addition of TRAIL. After 72 h, the cells were collected and resuspended in 500 μl of the ice-cold lysis buffer provided with the Caspases Assay kit (MLB International, Watertown, MA). After sonication, cell lysates were centrifuged and the resulting supernatants were analyzed for protein concentration by Bradford and stored at −20°C until further use. Colorimetric enzymatic activity assays for caspase-8 and caspase-3 were performed according to the manufacturer's instructions.

Transfection of c-FLIP and siRNA Oligonucleotides

The vector containing $c\text{-}FLIP_L$ was kindly provided by Dr. Roya Khosravi-Far, Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School (Boston, MA). OVCA 420 and OVCA 429 cell lines were plated at a density of 8×10^4 cells per well in six-well plates. After 24 h, the cells were transfected with 0.1 μg of the pcDNA3/Zeo vector alone or pcDNA3/Zeo vector containing $c\text{-}FLIP_L$ cDNA, using Lipofectamine Plus Transfection reagents (Invitrogen, Carlsbad, CA). Twenty-four hours post-transfection, cells were treated with TRAIL (33 ng/ml for OVCA 420, or 330 ng/ml for OVCA 429) for 3 days and harvested for RT-PCR and Western blot analysis. The overall transfection efficiency of OVCA cells as determined by X-Gal staining against pSV-beta-galactosidase-transfected cells was 66%. Highly purified siRNAs (Qiagen, Valencia, CA) targeted against

c-FLIP$_L$ (5′-AATTCAAGGCTCAGAAGCGAG-3′), or c-FLIP$_S$ (5′-AACACCCTATGCCCATTG-TCC-3′) were delivered into OVCA 432 cells with the TransMessenger Transfection Reagent (Qiagen) according to the manufacturer's instructions. After 24 h, cells were treated with 330 ng/ml TRAIL for 3 days. Total RNA and protein extractions were conducted for RT-PCR and Western blot, respectively.

Statistical Analysis

Results are expressed as the mean ± SD of at least three independent experiments. Data were analyzed by ANOVA, followed by Tukey's post-hoc test. Statistical significance was inferred at ($P < 0.05$).

RESULTS

HOSE and OVCA Cell Lines Exhibit Differential Sensitivity to TRAIL

To determine the sensitivity of immortalized normal ovarian surface epithelial (HOSE) cell lines and OCa cell lines to TRAIL-induced cell death, six cell lines were treated with increasing doses of TRAIL. We first examined the effects of TRAIL in inducing cell loss in four cancer cell lines (OVCA 420, OVCA 429, OVCA 432, and OVCA 433), and two normal cell lines (HOSE 12–12 and HOSE 642), using the MTS assay. Three out of four OCa cell lines were sensitive to TRAIL-induced cell loss in a dose-dependent manner (Fig. 1A). OVCA 420 cells were the most sensitive to TRAIL, exhibiting 80% cell death upon treatment with 33 ng/ml of TRAIL, whereas OVCA 429 and OVCA 433 cells were moderately sensitive, exhibiting 65% and 52% cell death, respectively, when exposed to 330 ng/ml of TRAIL. In contrast, OVCA 432 cells and the two HOSE cell lines were resistant to TRAIL-induced cell loss (Fig. 1A). We next examined whether TRAIL-induced cell loss is mediated via activation of an extrinsic apoptotic cascade involving activation of caspase-8 and -3 in all six cell lines. Analogous to TRAIL-induced cell death, activation of these two caspases was only observed in TRAIL-sensitive lines (Fig. 1B). The specificity of caspase-8- or caspase-3-activation in TRAIL-sensitive cell lines was established from the observation that specific inhibitors, IETD-fmk or DEVD-fmk, blocked the activation of their respective proteases (Fig. 1B).

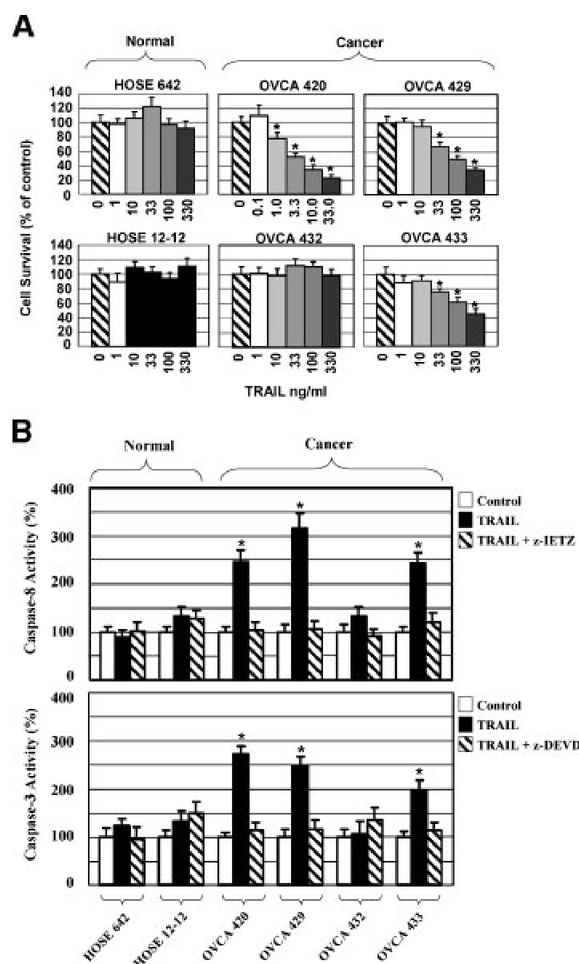

Fig. 1. Effect on TRAIL on ovarian cell lines. **A**: Differential sensitivity to TRAIL in normal (HOSE) and tumor (OVCA) cell lines. Four OVCA and two HOSE cell lines (1×10^3/well in 96 well-plate) were cultured for 3 days with different concentrations of recombinant human (rh) (TRAIL 0–33 ng/ml for OVCA 420, 0–330 ng/ml for others), and cell viability measured by MTS assay. Results represent the average of three experiments expressed as percentage of untreated controls (Mean ± SD, $*P < 0.05$). **B**: Effect of the caspase-3 inhibitor z-DEVD-fmk and the caspase-8 inhibitor z-IETD-fmk (10 μM) on TRAIL-induced apoptosis. HOSE and OVCA (2×10^5 cells in 25 cm^2 culture flasks) cells were treated with TRAIL (black bars, 33 ng/ml for OVCA 420 and 330 ng/ml for others), pretreated with z-DEVD-fmk or z-IEDT-fmk (10 μM) for 1 h and then treated with vehicle (open bars) or with TRAIL (hatched bars) for 3 days. Enzyme activities in cell lysates toward caspase substrates DEVD-pNA (caspase-3) or IETD-pNA (caspase-8) were measured in ovarian cells. Caspase activity in control cells is expressed as 100%. The figure shows the mean ± SD of triplicates from two independent experiments ($*P < 0.001$–0.02).

TRAIL Receptor Expression Does not Correlate With Sensitivity to TRAIL in OVCA Cells

To assess whether expression of death receptors and their decoys is linked to

TRAIL-sensitivity/resistance in OCa cell lines, we studied the expression of DR4, DR5, DcR1, and DcR2 by semiquantitative reverse transcription (RT)-polymerase chain reaction (PCR) as described before [Syed and Ho, 2003]. All death and decoy receptors were expressed at comparable levels among TRAIL-sensitive (OVCA 420, OVCA 429, and OVCA 433) and -resistant (OVCA 432 and HOSE) cell lines (data not shown), suggesting that the sensitivity of cell lines to TRAIL is not due to differences in the receptor expression levels.

Inverse Relationship Between c-FLIP$_L$ Expression and TRAIL Sensitivity in OVCA Cells

c-FLIP is an inhibitor of death receptor-mediated apoptosis, and is presumed to play an important role in modulating TRAIL sensitivity [Golks et al., 2005]. In this study, the expression of the two major forms of c-FLIP, long (c-FLIP$_L$) and short (c-FLIP$_S$) isoforms, was determined at the transcriptional level in the presence or absence of TRAIL in four OCa cell lines (Fig. 2A). All four cell lines expressed similar levels of c-FLIP$_S$, which were not affected by TRAIL treatment. In contrast, TRAIL-resistant OVCA 432 cells overexpressed c-FLIP$_L$ transcript when compared to TRAIL-sensitive cell lines. Furthermore, while treatment with TRAIL downregulated c-FLIP$_L$ mRNA in TRAIL-sensitive lines, it failed to do so in OVCA 432 cells. These data implicate that persistent overexpression of c-FLIP in OVCA cells is a basis for TRAIL resistance. We therefore proceeded to employ siRNAs to inhibit specifically the expression of c-FLIP$_L$, as well as that of c-FLIP$_S$ for comparison. Transfection of OVCA 432 cells with siRNAs against c-FLIP$_L$ or c-FLIP$_S$ reduced the levels of their respective transcripts (Fig. 2B) and proteins (Fig. 2C) in this cell line. However, only siRNA against c-FLIP$_L$ caused significant reduction in cell viability in OVCA 432 cells, whereas siRNA against c-FLIP$_S$ had no effect.

Overexpression of c-FLIP$_L$ Increases TRAIL Resistance in TRAIL-Sensitive OVCA Cells

To investigate if overexpression of c-FLIP$_L$ is able to reduce TRAIL sensitivity in sensitive cell lines, OVCA 420 and OVCA 429 cells were transfected with a vector containing c-FLIP$_L$ (Fig. 3). A marked increase in c-FLIP$_L$

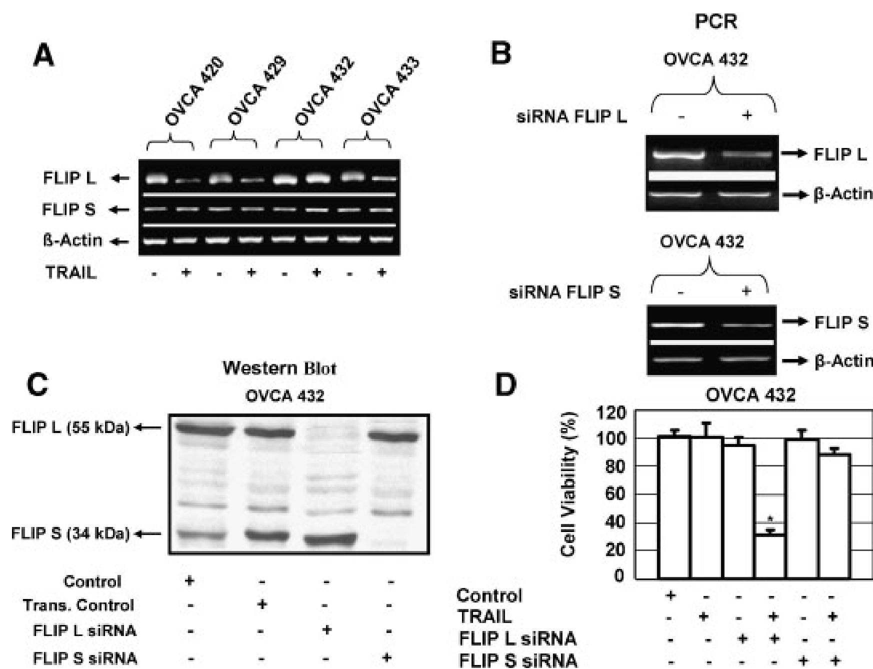

Fig. 2. Inhibition of c-FLIP$_L$ abolishes TRAIL resistance in OVCA 432. **A**: Basal and TRAIL-regulated (TRAIL, 33 ng/ml for OVCA 420 and 330 ng/ml for others) expression of c-FLIP$_L$ and c-FLIP$_S$ transcripts were detected in OVCA cell lines by RT-PCR. OVCA 432 cells were transfected with siRNA directed toward c-FLIP$_L$ or c-FLIP$_S$ for 24 h, (**B**) mRNA expression was examined by RT-PCR, and (**C**) protein expression was determined by Western blot using antibodies against c-FLIP$_L$, c-FLIP$_S$, or beta-actin for normalization. **D**: Cell viability was measured by MTS assay in OVCA 432 cells transfected with c-FLIP$_L$ or c-FLIP$_S$ siRNA after treatment with TRAIL (330 mg/ml) for 72 h. Data are expressed as the mean $\pm$ SD of three independent experiments (*$P < 0.001$).

expression was observed at both messenger and protein levels (Fig. 3A,B). Cells overexpressing c-FLIP$_L$ were treated with 33 (OVCA 420) or 330 (OVCA 429) ng/ml of TRAIL and proliferation was measured after 72 h. TRAIL failed to induce apoptosis in both OCa cell lines overexpressing c-FLIP$_L$ (Fig. 3C). Thus, transient overexpression of c-FLIP$_L$ is capable of changing the phenotype of OCa cells from TRAIL-sensitive to TRAIL-resistant.

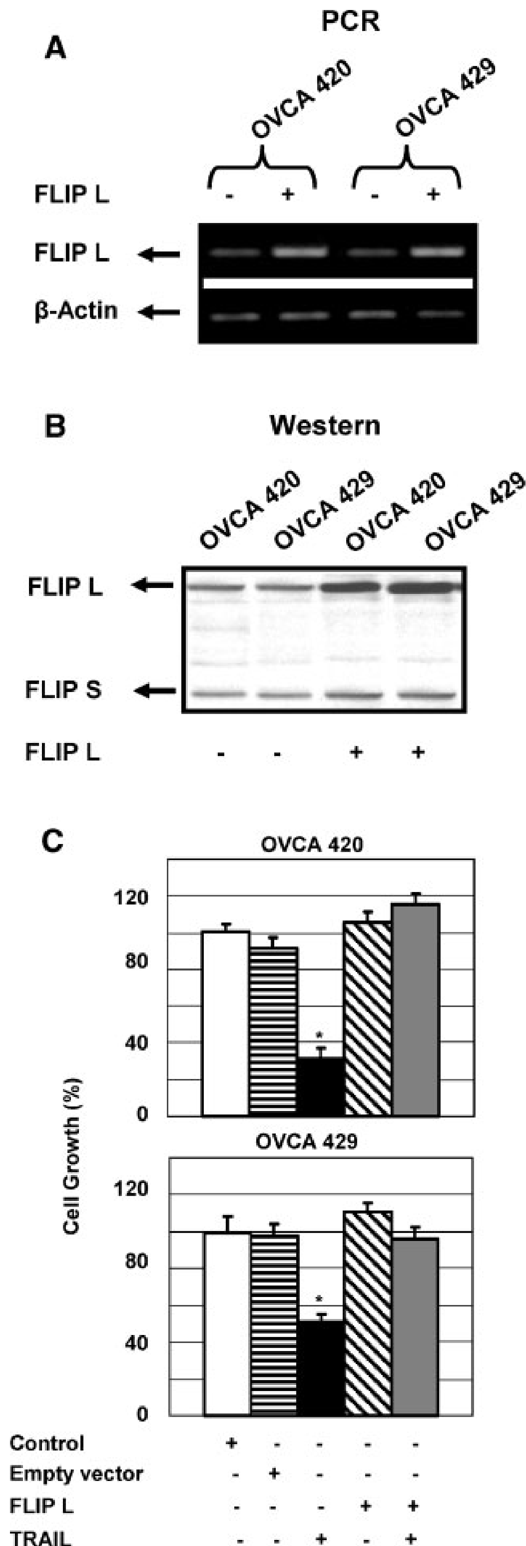

Fig. 3. Ectopic expression of c-FLIP$_L$ increases resistance to TRAIL in OVCA cell lines. TRAIL-sensitive OVCA cell lines were transfected with the empty vector pcDNA 3.1-Zeo (−), or with c-FLIP$_L$ vector (+) and (**A**) mRNA, and (**B**) protein expression, were examined by RT-PCR or Western blot, respectively. **C**: Cell viability of mock-transfected or c-FLIP$_L$–transfected OVCA cells was assessed after treatment with TRAIL (OVCA 420 with 33 ng/ml and OVCA 429 with 330 mg/ml) for 72 h. Data are expressed as the mean ± SD of three independent experiments performed in triplicates, (*$P < 0.002$).

Progesterone and TRAIL Induce Apoptosis Cooperatively only in TRAIL-Sensitive OVCA Cells

We previously demonstrated that progesterone induces apoptosis in both TRAIL-sensitive and TRAIL-resistant OVCA cells. We conjectured that a combination of progesterone and TRAIL might enhance growth inhibition in cancer cells. For this reason, we treated the four OCa cell lines with progesterone (10^{-6} M) and 33 (OVCA 420) or 330 (OVCA 429, OVCA 432, and OVCA 433) ng/ml of TRAIL simultaneously for 72 h, and measured cell viability by MTS assay (Fig. 4). Progesterone inhibited 60–70% of growth in all four cancer cell lines. TRAIL caused significant (45–55%) growth inhibition in the three TRAIL-sensitive OVCA lines, but not in TRAIL-resistant OVCA 432 cells. When the TRAIL-sensitive OVCA lines were exposed to TRAIL plus progesterone, the combination resulted in a cooperative growth inhibition (80–90%) compared to either agent alone. However, the response of OVCA 432 cells to TRAIL remained unchanged in the presence of progesterone, with the growth inhibitory effect attributable only to progesterone.

Progesterone Induces Apoptosis in TRAIL-Sensitive and TRAIL-Resistant OVCA Cells Without Altering c-FLIP Levels

After demonstrating that progesterone does indeed induce cell death in TRAIL-resistant OVCA 432 cells, which have constitutively high levels of c-FLIP$_L$, we examined whether progesterone action involved downregulation of c-FLIP$_L$. c-FLIP$_L$ and c-FLIP$_S$ mRNA levels

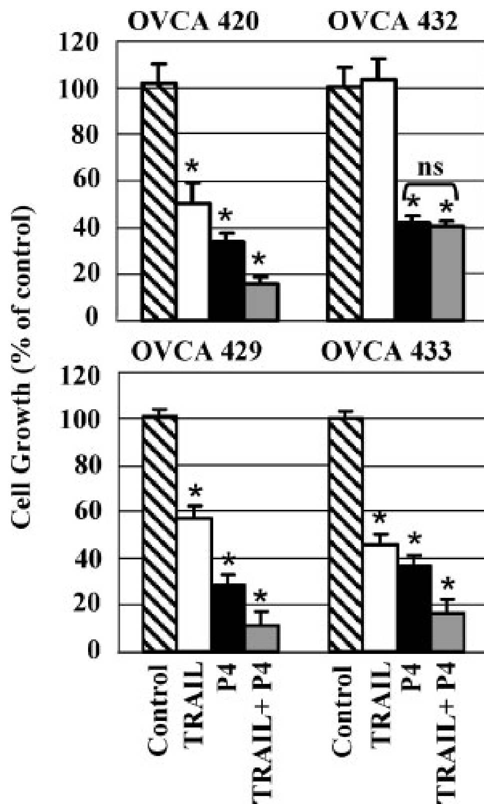

Fig. 4. Progesterone combined with TRAIL increases apoptosis in TRAIL-sensitive OVCA cells. Four OVCA cell lines were either treated with TRAIL (33 ng/ml for OVCA 420, 330 ng/ml for others), progesterone (10^{-6} M), or TRAIL plus progesterone for 3 days. Cell viability was determined by MTS assay. Data represent growth inhibition as a percentage of control cells. Results are expressed as the mean $\pm$ SD of three independent experiments (*$P < 0.001$–0.03).

were determined in four OCa cell lines in the presence/absence of 10^{-6} M progesterone (Fig. 5). Our results show that exposure of OVCA cells to progesterone did not alter the expression of either form of c-FLIP in both TRAIL-resistant and -sensitive cell lines.

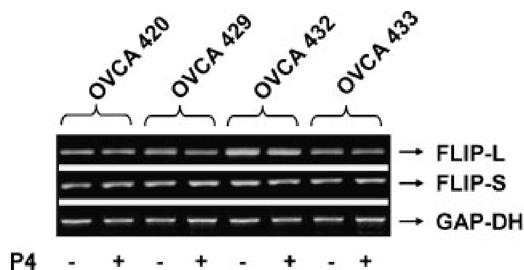

Fig. 5. Progesterone does not alter expression of c-FLIP$_L$ and c-FLIP$_S$ in OVCA cell lines. mRNA levels of c-FLIP$_L$ and c-FLIP$_S$ in four OVCA cell lines were detected by RT-PCR. A representative image from two independent experiments is shown.

Progesterone and MPA Synergistically Inhibit OVCA Cell Growth

To address the possibility of enhancing progesterone-induced OCa cell kill by addition of synthetic progestins, we compared the efficacy of each progestin, alone or in combination, on all four OCa cell lines. Singularly, progesterone, MPA, and NETA induced cell loss in these four cell lines in a dose-dependent manner (10^{-9}–10^{-6} M) (Fig. 6A). Efficacies are the following in descending

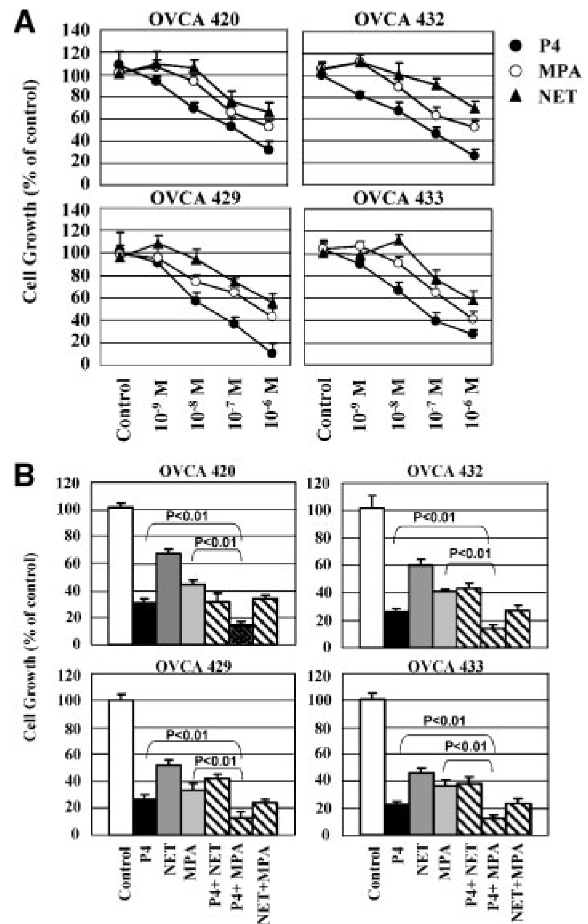

Fig. 6. Synergistic growth inhibition of OVCA cells by combination of progesterone and synthetic progestins. **A**: Four OVCA cell lines were incubated with increasing concentrations of progesterone, MPA or NETA for 3 days. Cell viability was measured by MTS assay. Data represent growth inhibition as a percentage of growth in control cells. **B**: Four OVCA cell lines were incubated with each progestagen (progesterone, MPA or NETA, 10^{-6} M) alone, or in combination for 3 days. Cell viability was measured by MTS assay. Data represent growth inhibition as a percentage of control cells. Results are expressed as the mean $\pm$ SD of at least three experiments. One-way ANOVA was applied to calculate the statistical significance among different groups (*$P < 0.01$).

order: progesterone > MPA > NETA. Progesterone was the most potent inducer of cell loss in OVCA cell cultures, causing about 80% cell reduction at a concentration of 10^{-6} M. When equal doses of progesterone, MPA or NETA were used in a dual regimen (Fig. 6B), progesterone plus MPA consistently produced the best results, causing over 85% cell reduction in TRAIL-sensitive and TRAIL- resistant (OVCA 432) cell lines.

DISCUSSION

The enduring progress in cancer treatment is mainly based on the combination of established cytotoxic compounds with new anticancer agents. This strategy aims to achieve a synergistic effect on tumor cell death, thereby improving clinical efficacy while reducing the potential for systemic toxicity. The objectives of the present study were: (a) to investigate the mechanism of TRAIL-induced apoptosis in four OCa cell lines; (b) to assess the responsiveness of OCa cells to different progestins approved for clinical use; and (3) to assess which combination of apoptosis-inducing agents would most favorably target all the OCa cell lines under study.

Our data demonstrate that TRAIL efficiently induces cell death (40–60% cell death) in three OCa cell lines (OVCA 420, OVCA 429, and OVCA 433), but fails to kill OVCA 432 cells (<10% death). Other studies have also described significant TRAIL-induced cytotoxicity (up to 80%) in OCa cells: three out of six (5), and one of three (7) OCa cell lines responded to TRAIL. Our results likewise supported the widespread postulation that TRAIL cytotoxicity is tumor-specific [Kelley and Ashkenazi, 2004], since normal HOSE cell lines were not affected by TRAIL in our experiment. Of significance, we found that TRAIL-induced cell loss in sensitive OCa cells is elicited by activation of caspase-8 and -3, suggesting the utilization of an extrinsic apoptotic pathway, a common mode of action for all TNF family ligands.

Potential mechanisms of resistance to TRAIL include, among others, overexpression of decoy receptors, cell-surface downregulation of DR4 and DR5, and upregulated expression of c-FLIP in the presence of TRAIL [Horak et al., 2005; Lane et al., 2006]. To investigate whether any of these mechanisms was responsible for TRAIL resistance in OVCA 432 cells, we examined TRAIL receptor expression in both sensitive and resistant cell lines. Death and decoy receptors were expressed in all cell lines at various degrees, although no consistent differences were observed between sensitive and resistant cells. This suggests that resistance in OVCA 432 cells cannot be explained by alterations in TRAIL receptor/decoy expression, but rather by differences in intracellular signaling molecules located downstream of the death receptors.

We next investigated if c-FLIP overexpression is responsible for TRAIL resistance in OVCA cells. At the mRNA level, multiple c-FLIP splice variants exist. However, only three protein isoforms—c-FLIP$_L$, c-FLIP$_S$, and c-FLIP$_R$—have been described [Golks et al., 2005]. All three isoforms have two death effector domains (DEDs), which are critical for their recruitment to the death inducing signaling complex (DISC). The two shorter forms, c-FLIP$_S$ and c-FLIP$_R$, block DR-induced apoptosis by inhibiting activation of procaspase-8 at the DISC. Yet, the role of c-FLIP$_L$ at the DISC remains controversial, with some reports describing c-FLIP$_L$ as an anti-apoptotic molecule [Mezzanzanica et al., 2004; Zhang et al., 2004; Sharp et al., 2005] and others as proapoptotic [Chang et al., 2002; Boatright et al., 2004]. We report here that basal c-FLIP$_L$ transcript levels were at least twofold higher in TRAIL-resistant OVCA 432 cells than in the sensitive cell lines, and that TRAIL failed to decrease c-FLIP$_L$ expression only in OVCA 432 cells. These findings support the existence of a connection between c-FLIP$_L$ and TRAIL-mediated resistance in OVCA cells. To demonstrate a causal relationship between c-FLIP$_L$ expression and TRAIL sensitivity, a c-FLIP$_L$ siRNA was used to transfect resistant OVCA 432 cells. A significant downregulation of c-FLIP$_L$ mRNA and protein levels was achieved, which was accompanied by a striking increase in TRAIL sensitivity in this resistant cell line. Parallel experiments with a c-FLIP$_S$ siRNA ruled out the participation of c-FLIP$_S$ in these processes. Conversely, ectopic overexpression of c-FLIP$_L$ in the TRAIL-sensitive cell lines OVCA 420 and OVCA 429 rendered them resistant to TRAIL-induced cell death. Collectively, these facts strongly implicate c-FLIP$_L$ as the isoform responsible for granting TAIL resistance to OVCA 432 cells. Our findings are in agreement with those of other groups

showing that overexpression of c-FLIP confers resistance to TRAIL, whereas induced loss of this molecule correlates with acquisition of TRAIL sensitivity. This has been proven for a variety of cells including a colon cancer cell line [Burns and El-Deiry, 2001], erythroleukemia cells [Hietakangas et al., 2003], human fibroblasts [Griffith et al., 1998], and a human carcinoma of the nasopharynx [Siegmund et al., 2002].

Although promising, the use of siRNA technology to downregulate c-FLIP as a therapeutic tool to overcome TRAIL resistance has to surmount ample limitations. We reasoned that exploiting treatments currently approved for other conditions, such as the natural hormone progesterone and synthetic progestins [Affandi, 2002; Stahlberg et al., 2004], might yield an earlier improvement in OCa management. We previously reported a strong proapoptotic effect of progesterone in OCa cells. Hence, we tested here whether combined treatment of OCa cells with progesterone and TRAIL could subdue TRAIL resistance. We observed a cooperative effect between progesterone and TRAIL in inducing apoptosis in OCa cells. However, this outcome was only observed in TRAIL-sensitive cells. Progesterone did not sensitize resistant OVCA 432 cells to TRAIL, but this hormone alone was able to induce significant cell death in them.

An important observation made in our study is that progesterone-induced apoptosis in both TRAIL-sensitive and -resistant OCa cell lines is independent of c-FLIP$_L$ downregulation. This finding suggests that progesterone could be exploited to treat TRAIL-resistant OCa cells in which overexpression of c-FLIP accounts for TRAIL resistance. Furthermore, a mainstay treatment for OCa is the use of cisplatin, and chemoresistance to cisplatin in OCa cells has also been linked to overexpression of c-FLIP. Knockdown of c-FLIP by siRNA resulted in sensitization to cisplatin-induced apoptosis [Abedini et al., 2004]. Thus, it is logical to expect that treatment with progesterone may be a good option for OCas in which resistance to apoptotic-inducing agents could be attributable to c-FLIP overexpresssion.

As all OCa cell lines, including OVCA 432, had a good response to progesterone, experiments were then designed to evaluate the efficacy of synthetic progestins already in clinical use, such as MPA and NETA. We demonstrated that both MPA and NETA, like progesterone, induced cell death in a dose-dependent manner and regardless of TRAIL sensitivity, in the four OCa cell lines. The best apoptotic response was observed upon treatment with progesterone, followed by MPA and NETA. Three different combinations of progestins (progesterone + MPA, progesterone + NETA, and MPA + NETA) were also assayed. A cooperative effect between progesterone and MPA was observed with cotreatment achieving >85% cell kill for all four cell lines. Although the principal action of natural and synthetic progestins is mediated via the progesterone receptor (PGR) [Schindler et al., 2003], these compounds exhibit variable degrees of promiscuity toward transactivation of androgen (AR), glucocorticoid (GR), and mineralocorticoid (MR) receptors [Winneker et al., 2003]. Furthermore, the affinity of MPA for the PGR has been reported to be higher than that of progesterone [Sitruk-Ware, 2004]. Recent studies have demonstrated differential effects of progestins in human endothelial cells [Simoncini et al., 2004] and neuronal cells [Nilsen and Brinton, 2002, 2003]. Given this evidence, it is not surprising for us to find maximum anti-apoptotic efficacy in OCa by combined treatment with progesterone and MPA.

In conclusion, our study demonstrates that progesterone is a more potent death-inducing agent than TRAIL for OCa cells, and that it cooperates with the anti-apoptotic action of this TNF family ligand in TRAIL-sensitive cells. TRAIL-initiated apoptosis relies on down-regulation of c-FLIP$_L$ levels with failure to reduce c-FLIP$_L$ accounting for TRAIL resistance in OCa cells. Importantly, progesterone-induced apoptosis was shown to occur in both TRAIL-sensitive and -resistant OCa cells, and thus it is not limited by the anti-apoptotic action of c-FLIP$_L$. Furthermore, the widely used progestins MPA and NETA are both effective as anti-OCa agents when used alone, and even more in combination with progesterone. The optimal anti-OCa outcome was attained by a combination of progesterone and MPA, not only in TRAIL-sensitive cells, but also in TRAIL-resistant ones. These findings provide a strong rationale for clinical trials to test the efficacy of combined therapies using progestins with other mainstay (cisplatin) or developmental (TRAIL) agents.

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