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Apoptosis Induction in Prostate Cancer Cells and Xenografts by Combined Treatment with Apo2 Ligand/Tumor Necrosis Factor-related Apoptosis-inducing Ligand and CPT-11[1]

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ABSTRACT

Because apoptosis is deregulated in most cancers, apoptosis-modulating approaches offer an attractive opportunity for clinical therapy of many tumors, including that of the prostate. LNCaP-derived C4-2 human prostate cancer cells are quite resistant to treatment with Apo2 ligand (Apo2L) or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), when using a nontagged, Zn-bound recombinant trimeric version that is devoid of any exogeneous sequences and therefore least likely to be immunogenic in human patients and that has been optimized for maximum efficacy and minimum toxicity. When combined with the topoisomerase I inhibitor CPT-11 (irinotecan), Apo2L/TRAIL exhibits enhanced apoptotic activity in C4-2 cells cultured *in vitro* as well as xenografted as tumors *in vivo.* Apoptosis both *in vitro* and *in vivo* was characterized by two major molecular events. First, apoptosis induction was accompanied by changes in expression levels of the Bcl-2 family genes and their products. However, whereas combination treatment applied to *in vitro* cell culture was characterized by a significant up-regulation and activation of Bax and down-regulation of Bcl-xL, the treatment applied to tumors induced Bak and Bcl-xS, whereas Bcl-ω and Bcl-xL were down-regulated. Because there are multiple members of the Bcl-2 family (24 members to date), these data indicate that, under different biological conditions, different proteins may be responsible for activating apoptosis and provide evidence for a differential regulation of the multidomain Bcl-2 protein-encoding genes, *bax* and *bak.* Increased Bax expression led to its activation, translocation to the mitochondria, and release of cytochrome *c.* In addition, this combination treatment induced apoptosis through potent activation of caspase-8 and the proapoptotic protein Bid, resulting in activation of effector caspase-3 and cleavage of its cellular target protein, poly(ADP-ribose) polymerase (PARP), events blocked by the pan-caspase inhibitor *N*-tert-butoxy-carbonyl-Val-Ala-Asp-fluoro methylketone (zVAD-fmk). Activation of multiple caspases and PARP cleavage were also observed in the C4-2 tumors treated with doses resulting in effective tumor control at 42 days after Apo2L/TRAIL plus CPT-11 treatment. Down-regulation of Bax by small interference (RNA) (siRNA) in C4-2 cells significantly prevented PARP cleavage and apoptosis. Strikingly, similar experiments in cells stably expressing a dominant-negative death receptor DR5 led to complete ablation of PARP cleavage and apoptosis, indicating the essential role of both mitochondrial and receptor-mediated apoptotic pathways. Our data indicate that the combined treatment of Apo2L/TRAIL and CPT-11 achieves tumor control in prostate cancer tumors through regulation of Bcl-2 family proteins and potent activation of caspases.

INTRODUCTION

Prostate cancer has become the most frequently diagnosed, noncutaneous neoplasm and the second leading cancer-related death in the United States (1). Therefore, novel therapeutic strategies for treatment of prostate cancer are urgently needed. C4-2 is an androgen-independent subline of LNCaP, an androgen-dependent human prostate cancer cell line (2). Like LNCaP, C4-2 cells secrete prostate-specific antigen and express prostate-specific membrane antigen, prostatic acid phosphatase, and functionally active androgen receptor. Unlike LNCaP, C4-2 cells are androgen refractory, highly tumorigenic, and metastatic in a castrated host (3). Therefore C4-2 cells could serve as a valuable model of human prostate cancer.

Apo2L[3]/TRAIL is one of several members of the tumor necrosis factor gene superfamily that induce apoptosis through engagement of DRs (4, 5). Apo2L/TRAIL is expressed as a type 2 transmembrane protein, which forms a homotrimer that binds three receptors, each at the interface between two of its subunits (6, 7). The presence of a cysteine-bound Zn atom in the trimeric ligand is considered to be essential for optimal biological activity (8, 9), and therefore the method of preparing recombinant soluble ligand is important for its optimal biological activity. Some Apo2L/TRAIL preparations lacking Zn have reduced solubility and tend to aggregate, which might explain the reported toxicity of such preparations to human hepatocytes (10, 11). In contrast, the untagged, trimeric Apo2L/TRAIL that contains stoichiometric Zn has no cytotoxic effect on human or nonhuman primate (cynomolgus monkey and chimpanzees) hepatocytes (11) or keratinocytes (12).

Apo2L/TRAIL induces apoptosis selectively in a variety of tumor cells, but not in normal cells or tissues, a property that makes it an excellent candidate therapeutic for treating cancer patients (4, 5, 13–15). Apo2L/TRAIL induces apoptosis in a variety of human cancers, such as colon, lung, breast, prostate, pancreatic, kidney, central nervous system, and thyroid cancers, leukemia, and multiple myeloma. Its effect is independent of p53 status, and thus it might be very useful for treating a large range of tumors, including those deficient in p53 function (16). In athymic or severe combined immunodeficient mice bearing human tumor xenografts derived from colon carcinoma (17, 18), breast carcinoma (13), multiple myeloma (19), or glioma (20, 21), administration of recombinant soluble Apo2L/TRAIL shows a significant antitumor activity without systemic toxicity. Moreover, in combination with other DNA-damaging agents (17, 22) or radiation (23, 24), Apo2L/TRAIL has synergistic antitumor activity both *in vitro* and *in vivo* in xenograft mouse models. One such agent is irinotecan (CPT-11), a water-soluble prodrug derivative of camptothecin. It is a DNA topoisomerase I inhibitor and is believed to block DNA transcription and replication through the inhibition of this enzyme. CPT-11 is currently used as a first- and second-line therapy in the treatment of patients with advanced colorectal cancer (25). Recently, CPT-11 was shown to augment Apo2L/TRAIL-induced apoptosis in cell lines of breast cancer, colon carcinoma, and glioma (17, 22, 26–28).

Two main signaling pathways initiate apoptosis in mammalian cells: the cell-extrinsic and cell-intrinsic pathways. Apo2L/TRAIL

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[3] The abbreviations used are: Apo2L, Apo2 ligand; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; PARP, poly(ADP-ribose) polymerase; DR, death receptor; DcR, decoy receptor; siRNA, small-interference RNA; DEVD, Asp-Glu-Val-Asp; pNA, *p*-nitroanilide; zVAD-fmk, *N*-tert-butoxy-carbonyl-Val-Ala-Asp-fluoromethylketone; RPA, RNase protection assay.

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can activate the cell-extrinsic pathway by binding to five members of the tumor necrosis factor receptor family, DR4 (TRAIL-R1), DR5 (TRAIL-R2), DcR1, DcR2, and osteoprotegerin (16). This pathway of apoptosis is independent of the p53 status of the cells. Both DR4 and DR5, type I transmembrane proteins known to mediate apoptotic function, contain a conserved cytoplasmic death domain required for transducing the death signal. Two additional receptors, DcR1 and DcR2, have no or incomplete, truncated cytoplasmic death domains and act as DcRs by competing with DR4 and DR5 for Apo2L/TRAIL binding and thus inhibiting Apo2L/TRAIL-induced apoptosis (14, 29). Binding of Apo2L/TRAIL to DR4 and DR5 leads to recruitment of Fas-associated death domain and initiator procaspase-8 and procaspase-10 and thus results in their activation through proteolytic cleavage (30, 31), which in turn may activate downstream effector caspases, such as caspase-3, -6, and -7 (32). These activated effector caspases then proteolytically cleave a number of cellular proteins, *e.g.*, PARP (33), resulting in apoptosis. The cell intrinsic pathway triggers apoptosis primarily in response to DNA damage, such as that caused by radio- and chemotherapeutic agents (32, 34). This pathway often involves activation of p53, which, in turn, activates different proapoptotic Bcl-2 family proteins such as, Bax, Bak, PUMA, and NOXA (35). Bcl-2 family proteins are important regulators of apoptosis, with some members (Bcl-2, Bcl-xL, Bcl-ω, and Mcl-1) functioning as suppressors of apoptosis, and others (Bax, Bak, Bcl-xS, and Bid) functioning as promoters of cell death (35). The relative ratios of these various pro- and antiapoptotic members (*i.e.* homodimers:heterodimers) of the Bcl-2 family, rather than the expression level of any single Bcl-2 family protein, have been shown to determine the ultimate apoptotic sensitivity or resistance of cells to diverse stimuli (36).

When Bax is activated, it translocates to the mitochondria, causing membrane damage and resulting in the release of apoptogenic factors, such as cytochrome *c* and SMAC/DIABLO (37). In the cytosol, cytochrome *c* and dATP bind to Apaf-1 and cause its oligomerization (38). Apaf-1 then binds and activates caspase-9, which, in turn, activates the effector caspases (38, 39) and thereby initiates apoptosis. A cross-talk between these two pathways is mediated by the proapoptotic Bcl-2 family protein Bid, which is cleaved and activated by caspase-8. Active Bid then further activates Bax or Bak and thus amplifies apoptosis induction through the cell-intrinsic pathway. In some cell lines, death receptor (DR) DR engagement of the cell-extrinsic pathway is sufficient to induce apoptosis; however, in many cell types, apoptosis requires amplification of the cell-extrinsic pathway through the cell-intrinsic pathway (28, 40).

In the present study, we examined the role of non-tagged, Zn-bound soluble trimeric Apo2L/TRAIL and CPT-11 toward the synergistic cytotoxic killing of C4-2 prostate cancer cells and tumors. *In vitro*, Apo2L/TRAIL, when combined with CPT-11, induced activation of both the intrinsic and extrinsic apoptotic pathways. *In vitro*, this treatment caused changes in protein expression levels of the Bcl-2 family members Bax, Bcl-xL, and Bid and receptor-mediated signaling components. Activation of Bax, Bid, caspase-3, and -8 also played an important role. A similar activation of caspases, as well as induction of Bak, took place in C4-2 tumors treated with Apo2L/TRAIL plus CPT-11 at doses resulting in effective tumor control. Bax and Bak were induced sequentially in tumors, with additional changes observed for Bcl-xS, Bcl-ω, and Bcl-xL. Down-regulation of Bax by siRNA and of DR5 by a dominant-negative mutant completely prevented PARP cleavage and apoptosis. These data indicate that the combined treatment of Apo2L/TRAIL plus CPT-11 induces apoptosis through potent activation of Bcl-2 family proteins and caspases to achieve tumor control.

MATERIALS AND METHODS

Materials. The recombinant human soluble trimeric Apo2L/TRAIL (11, 17) was a gift from Genentech, and CPT-11 (irinotecan hydrochloride) was from Pharmacia and Upjohn Co. (Kalamazoo, MI). Apo2L/TRAIL and CPT-11 were used at a concentration of 100 ng/ml, unless otherwise indicated. All the chemicals, unless otherwise specified, were obtained from Sigma (St. Louis, MO).

Cell Culture. C4-2 human prostate cancer cells (2) were obtained from Dr. W. Heston (Cleveland Clinic) and cultured in RPMI 1640 plus 10% fetal bovine serum supplemented with 100 μg/ml streptomycin, 100 units/ml penicillin, 2 mM glutamine, and 250 μg/ml fungizone (Invitrogen, Carlsbad, CA). All cells were maintained in culture at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Blocking Apoptosis Signaling. siRNA for the *bax* gene was prepared following the recommendations from the siRNA construction kit and manufacturer (Ambion). The primer was 5′-AAC-ATG-GAG-CTG-CAG-AGG-ATG-A-CCTGTCTC-3′, with the *underlined sequence* matching the corresponding sequences in the *Bax* mRNA. Transfection of siRNA for targeting the endogeneous *bax* gene was carried out using siPORT lipid (Ambion) and a final siRNA concentration of 100 nM. Specific silencing of targeted genes was confirmed by at least three independent experiments. C4-2 cells were also transfected with pcDNA3-DR5Δ (residues 1–268; Ref. 41), as described previously (23, 42). ΔDR5 lacks the death domain and has been shown to function as a dominant-negative mutant, inactivating the function of the endogenous DR5 (41). ΔDR5 contains a FLAG epitope tag, which facilitates examination of its expression levels. Transfected cells were selected in the presence of 1 mg/ml G418 (Invitrogen)-containing media and subsequently maintained with 0.5 mg/ml G418.

Apoptosis and Colony Regression Assays. Apoptosis assays were performed, as described previously (43), using Hoechst 33258 (10 μg/ml). At the end of each treatment, all cells (suspension and trypsinized) were pooled and washed with PBS, and an aliquot was incubated with Hoechst 33258 for 5 min. The incidence of apoptotic chromatin changes was determined by counting and scoring 250 cells/experimental sample under UV fluorescence microscopy. Each experiment was performed using three replicated wells for each drug concentration and carried out independently at least three times.

For colony regression assays, C4-2 cells (100 cells/dish) were grown in 60-mm dishes for 2 weeks before treatment for 4 h with Apo2L/TRAIL and CPT-11, alone or in combination. Then cells were washed with and further incubated in drug-free media for another week. Colonies were fixed in 70% ethanol, stained with crystal violet [0.4% in 95% ethanol (44)] and counted with an automatic colony counter (Image-Pro Plus version 4.5.0.22).

Caspase Assays. Caspase activity was determined, as described previously (23, 42, 45), using acetyl-DEVD-pNA (Biomol, Plymouth Meeting, PA), a preferred substrate for caspase-3 and -7, by the enzyme-catalyzed release of pNA monitored at 405 nm in a microtiter plate reader (Cambridge Tech, Inc., Medford, MA). For *in vivo* caspase inhibition studies, the tetrapeptide pan-caspase inhibitor zVAD-fmk (50–100 μM; Biomol) was added 30 min before treatment with Apo2L/TRAIL and CPT-11 and remained in the medium until cell lysis for caspase assays (6 h). For *in vitro* caspase inhibition, zVAD-fmk was added (50–100 nM) to apoptotic cell lysates (prepared at 6 h after the treatments) for 30 min at 37°C before incubation with the DEVD-pNA substrate. Substrate only was used for background control, and the pNA-derived caspase substrate cleavage activity was normalized against untreated cells.

Immunocytochemistry. C4-2 cells were grown on glass coverslips and treated with Apo2L/TRAIL and CPT-11, alone or in combination, for 4 h and fixed in 4% paraformaldehyde for 20 min at room temperature. The coverslips were washed twice with PBS, and nonspecific binding was blocked using 2% goat serum with 0.3% Triton X-100 for 10 min. Cell monolayers were then incubated with antibodies to active caspase-3, active caspase-7 [rabbit polyclonal antibody (1:100); Cell Signaling Technology, Beverly, MA], active Bax [mouse monoclonal antibody 6A7 (1:250) against amino acids 12–24 of Bax; PharMingen], and cytochrome *c* [mouse monoclonal antibody (1:100); PharMingen] in blocking serum for 1.5 h. After washing twice with PBS, cell monolayers were incubated with an Alexa Fluor 488-conjugated secondary antibody (Molecular Probes, Eugene, OR) for 1 h at room temperature in the dark. The coverslips were washed twice in PBS, mounted with 4′,6-diamidino-2-phenylindole (DAPI) containing Vectashield (Vector Laboratory, Burl-

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ingame, CA), and observed under a confocal fluorescence microscope (Leica TCS-SP2, Heidelburg, Germany). MitoTracker Red (400 nM; Molecular Probes) was used before fixation for mitochondria localization (43).

RPA. RNAs were isolated using Trizol reagent (Invitrogen) from C4-2 cells or tumor tissues isolated from mice treated with Apo2L/TRAIL, CPT-11, or a combination of both. The steady-state mRNA expression was determined using the RiboQuant system (PharMingen) for RPA (23). The hApo2b, hAPO1c, hAPO3c, and hStress-1 multiprobe templates sets (PharMingen) were used for the T7 polymerase-directed synthesis of high specific activity ^{32}P-labeled antisense RNA probes. The levels of each mRNA species were determined by PhosphorImager (Molecular Devices, Sunnyvale, CA) analysis based on signal intensities given by the appropriately sized, protected probe fragments, which were also normalized to the expression levels of the housekeeping gene, L32, and to the levels of each mRNA found in control, untreated cells.

***In Vivo* Analysis of Antitumor Activity of Apo2L/TRAIL and CPT-11.** Athymic (*nu/nu*) 6-week-old male mice (Taconic Farms, Germantown, NY) were used for all *in vivo* experiments. Mice were maintained under pathogen-free conditions in laminar flow boxes in accordance with established institutional guidance and approved protocols. All mice were immunosuppressed by irradiating them with 3 Gy. The next day, they received s.c. injection in the left flank with 3×10^6 C4-2 cells (in 250 μl of Matrigel matrix; BD Bioscience). After the xenografts reached ~100 mm^3 in size (~12 days), tumor-positive animals were randomly sorted into treatment groups, and the average tumor size for each group was determined. The value for each group was set to 0%, and all subsequent changes in tumor size for each group were expressed as a percentage change in comparison with the starting tumor mass. Apo2L/TRAIL (5 consecutive days, on days 13–17), CPT-11 (3 days, on days 12, 16, and 20), or vehicle control (TBS) was administered by i.p. injection (200 μl in TBS). For the Apo2L/TRAIL plus CPT-11 treatment, mice were divided into two groups. After the second week, one group received no further treatment, and the second group was subjected to injections with Apo2L/TRAIL (5 consecutive days, on days 28–32). Growth curves were performed by externally measuring tumors twice/week. Percentage changes in tumor size were calculated as follows: [(Tumor size posttreatment) − (Tumor size at day 12)/(Tumor size at day 12)] × 100%. Weight of mice was measured twice/week for the duration of the experiments. For detection of apoptosis, mice were sacrificed at 20 h after the first injection (day 13) and at 4 h after the last injection (day 17). The xenografts were instantly frozen in liquid nitrogen and stored for further analysis.

Immunohistochemistry. Formalin-fixed and paraffin-embedded mice tissue sections were examined for active caspase-3, caspase-7, and expression of Bak using the Ventana ES Autostainer and 3,3′-diaminobenzidine kits (Ventana Medical Systems, Tucson, AZ). The slides were deparaffinized with xylin and graded alcohol and treated with citrate buffer (pH 6) for 20 min for antigen retrieval. The slides were incubated with primary antibodies to active caspase-3, active caspase-7, and Bak (1:100 dilution) for 32 min on the Ventana ES Autostainer. Sections were counterstained with hematoxylin and examined under the microscope, with representative areas being photographed using a ×20 objective.

Western Blotting. Cell or tissue lysates (50 μg in buffer containing 1% NP40, 20 mM HEPES, 4 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml trypsin inhibitor, 5 mM benzamidine, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin) were separated by SDS-PAGE and electrotransferred onto nitrocellulose membranes [Schleicher and Schull, Keene, NH (23, 43, 45)]. Blots were blocked with 5% nonfat dry milk in 0.1% Tween 20 in PBS for 1 h at room temperature and incubated overnight at 4°C with primary antibodies for Bid, Bak (PharMingen), caspase-3 p20, caspase-8 H277, caspase-9 p10, and Bax (Santa Cruz Biotechnology), PARP (Biomol), and Flag M2 and β-actin (Sigma), according to the manufacturer's recommended dilution, followed by incubation with secondary horseradish peroxidase-conjugated antibodies (Amersham Biosciences) for 1 h at 37°C. β-Actin was used as an internal standard for protein loading. Immunoreactive bands were visualized by enhanced chemiluminescence and subsequent exposure to hyperfilm (X-ray film; Eastman Kodak).

RESULTS

The Combined Apo2L/TRAIL and CPT-11 Treatment Acts Synergistically to Induce Apoptosis in C4-2 Cells. Our previous studies have indicated that C4-2 prostate carcinoma cells were quite resistant to the effect of trimeric Apo2L/TRAIL (11). However, they became quite sensitive to Apo2L/TRAIL when it was used together with inhibitors of transcription or translation. Experiments performed with a number of chemotherapeutic agents that had been used previously in combination with Apo2L/TRAIL in other tumor models (17) revealed that CPT-11 was quite effective as an addition for Apo2L/TRAIL treatment.[4] To further assess the effect of Apo2L/TRAIL, alone or in combination with CPT-11, we examined nuclear condensation using Hoechst 33258 staining as a standard parameter used to evaluate apoptosis. When C4-2 cells were incubated with CPT-11 (100 ng/ml) and Apo2L/TRAIL (11–300 ng/ml), most of the cells did dramatically undergo apoptosis. This effect was dose dependent (Fig. 1*A*), with a concentration of Apo2L/TRAIL as low as 11 ng/ml resulting in 40% viable cells, and with only 10% of C4-2 cells remaining viable when a concentration of 100 ng/ml was used. These results indicate a synergistic effect of the Apo2L/TRAIL plus CPT-11 treatment in C4-2 cells. Morphological changes of apoptosis, assessed as nuclear and chromatin condensation, were seen by fluorescence microscopy in the combined treatment as compared with single treatment (Fig. 1*B*). Because a concentration of 100 ng/ml for both Apo2L/TRAIL and CPT-11 was quite effective, resulting in a dra-

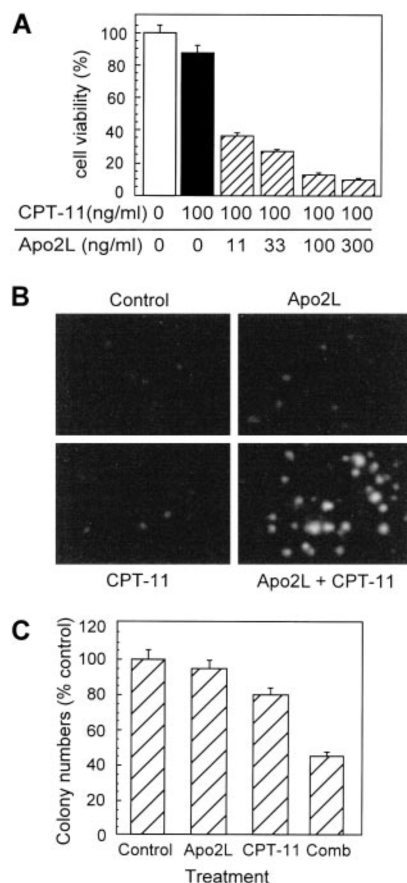

Fig. 1. Synergistic cytotoxic effect of Apo2L/TRAIL combined with CPT-11 on C4-2 cells. *A*, Cell viability was determined using Hoechst staining (10 μg/ml) after treating the cells with different concentrations of Apo2L/TRAIL (11–300 ng/ml) plus CPT-11 (100 ng/ml) for 48 h. Cell viability was expressed as a percentage of control cells. Data points represent the means of at least three different experiments; *bars*, SD. *B*, morphological changes in the nuclear chromatin by Hoechst staining (10 μg/ml) were visualized by fluorescence microscopy after treating cells with Apo2L/TRAIL (100 ng/ml), CPT-11 (100 ng/ml), and the combination for 48 h. *C*, inhibition of growth of colonies of C4-2 cells by the combination of Apo2L/TRAIL plus CPT-11 was seen in an *in vitro* colony regression assay as described in "Materials and Methods."

[4] Unpublished observations.

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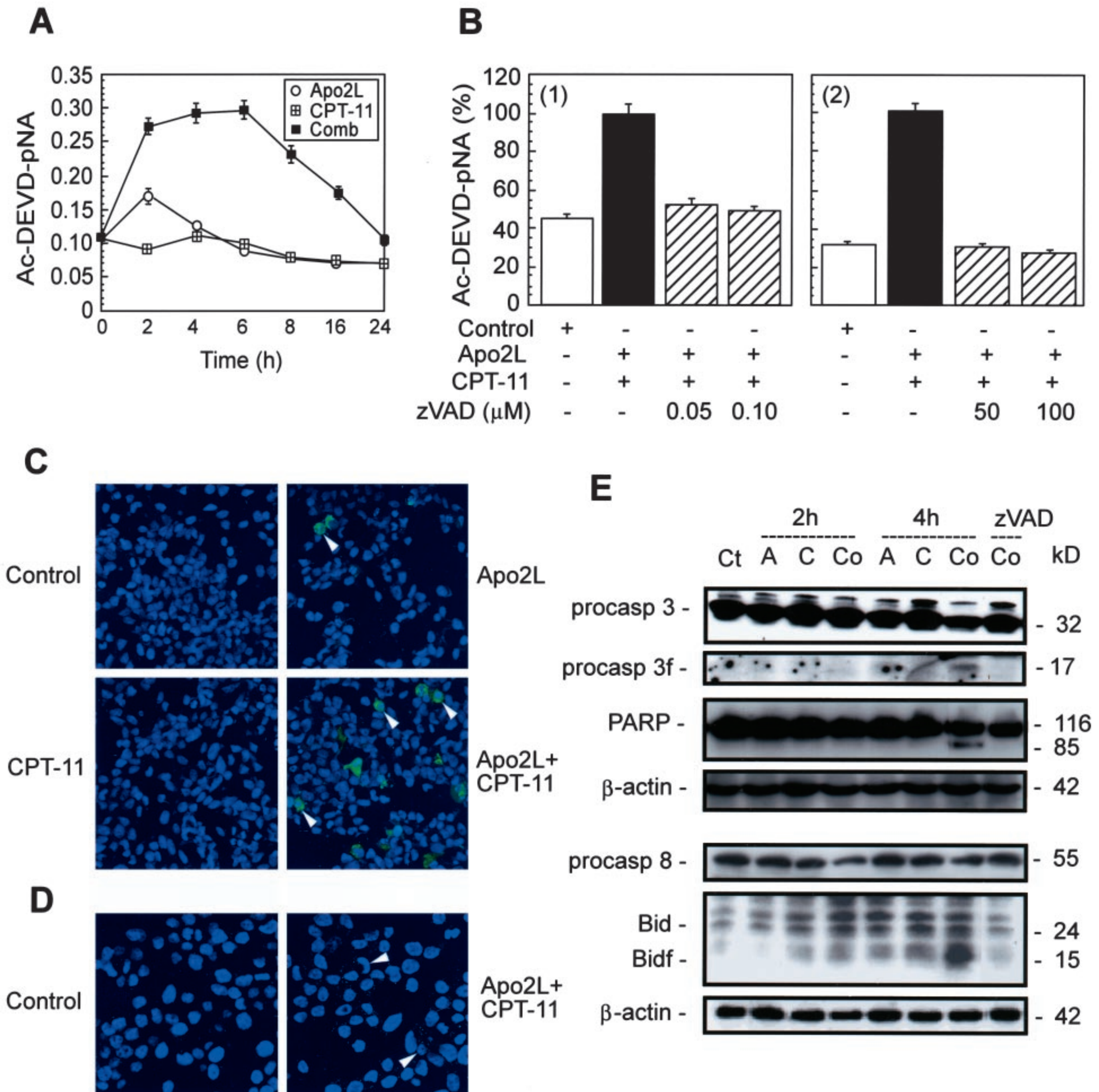

Fig. 2. Enhanced caspase activation by Apo2L/TRAIL plus CPT-11 in C4-2 cells. *A*, cells were lysed at the indicated times after treatment with Apo2L, CPT-11, and the combination. Cell lysates (100 μg of protein) were used for caspase-3 and -7 assays with Ac-DEVD-pNA (100 μM). *B*, for the caspase inhibition assay, cell lysates were collected at 6 h after treatment with Apo2L/TRAIL plus CPT-11. Cell lysates were incubated with zVAD-fmk (50–100 nM) *in vitro* for 30 min before the addition of the Ac-DEVD-pNA substrate (*1*), or cells were pretreated with zVAD-fmk (50–100 μM) for 30 min followed by exposure to Apo2L/TRAIL plus CPT-11 (*2*). The cleavage activities were determined colorimetrically at 405 nm. Values represent means ± SD ($n = 3$). *C*, enhanced active caspase-3 immunoreactivity was detected in the combination-treated in C4-2 cells as compared with control, Apo2L/TRAIL-, or CPT-11-treated cells by immunocytochemical staining using active caspase-3 antibody. Representative areas were photographed using a ×20 objective. *D*, enhanced active caspase-7 immunoreactivity was also detected in Apo2L/TRAIL plus CPT-11 treatment (representative areas were photographed using a ×40 objective). *E*, immunoblots were performed using the same cell lysates (100 μg of protein), as above, with anti-caspase-3, anti-caspase-8, anti-Bid, and anti-PARP antibodies, and β-actin was used as an internal control. Inhibition of caspase-3, caspase-8, Bid, and PARP cleavage was examined by using the pan-caspase inhibitor zVAD-fmk (50 μM) added to the cell monolayer 30 min before addition of Apo2L/TRAIL plus CPT-11, and incubated for another 4 h. Ct, A, C, and Co represent control Apo22 CPT-11, CPT-4, and combination, with arrowheads indicating active caspase-3 or 7 staining.

matic apoptotic effect in most cells, this dose was used for all further cell culture experiments.

To further assess the cytotoxic effect of Apo2L/TRAIL and CPT-11, a colony regression assay was performed. Treatment of established C4-2 colonies with Apo2L/TRAIL, CPT-11, and the combination resulted in a dramatic reduction in the number and size of colonies. The number of colonies in the combination treatment was 44.4% compared with 94.04%, 79.2%, and 100% in single treatments with Apo2L/TRAIL, CPT-11, or control untreated dishes, respectively (Fig. 1*C*). The clonogenic cell survival assay indicated similar results.[4]

Activation of the Intrinsic and Extrinsic Apoptotic Pathways. Caspase activation is the final common molecular event required for execution of apoptosis in most biological systems (32). To examine caspase activation, cell lysates obtained after treatment of C4-2 cells with Apo2L/TRAIL, CPT-11, and the combination were incubated with the chromogenic substrate DEVD-pNA, a preferred substrate for caspase-3 and -7. There was a time-dependent increase in DEVD-pNA cleavage activity in combination treatment as compared with single treatments (Fig. 2*A*). The increase in DEVD-pNA cleavage activity started at 2 h and reached a higher level at 6 h, indicating that apoptosis induced by Apo2L/TRAIL plus CPT-11 was dependent on

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caspase-3 and/or caspase-7 protease activity. To further examine the involvement of caspases in Apo2L/TRAIL plus CPT-11-induced apoptosis, we tested the effect of the pan-caspase inhibitor zVAD-fmk (0.05–100 μM) on DEVD-pNA cleavage activity *in vitro* (Fig. 2*B*, 1) as well as in cell culture (Fig. 2*B*, 2). In both cases, zVAD-fmk completely blocked combination treatment-induced caspase-3 and/or caspase-7 activation.

It has been reported that caspase-7 is predominantly activated during apoptosis of some prostate cancer cells (46). Because the caspase assays described above cannot distinguish between caspase-3 and -7 activation, immunocytochemical staining was next performed to determine which of the two caspases was activated. An antibody recognizing only the active form of caspase-3 was first used. This antibody, which does not recognize the proform of caspase-3, showed enhanced active caspase-3 immunoreactivity in the Apo2L/TRAIL plus CPT-11 treatment compared with control or single agent (Fig. 2*C*). The pan-caspase inhibitor zVAD-fmk (50 μM) blocked this reactivity (data not shown). Similarly to caspase-3, active caspase-7 was also detected in the combination treatment with Apo2L/TRAIL and CPT-11, but not in the control, untreated cells (Fig. 2*D*). However, the staining for caspase-7 was much weaker than that for caspase-3, indicating a lower level of expression. Parallel control experiments with untreated control cells, treated with the secondary antibodies only, showed no fluorescence (data not shown). Moreover, immunoblot analyses showed that caspase-3 was converted to its p17 active form derivative at the 4 h time point in the combination treatment, which was also blocked by zVAD-fmk (50 μM). By contrast, there was no active p17 cleaved product in the Apo2L/TRAIL or CPT-11 treatments (Fig. 2*E*). Previous studies (33) have identified a cellular substrate PARP as a nuclear apoptotic landmark, which is cleaved by active caspase-3 during apoptosis. Indeed, PARP was cleaved to the M_r 85,000 COOH-terminal fragment at 4 h of Apo2L/TRAIL plus CPT-11 treatment (Fig. 2*E*), which was also blocked by zVAD-fmk (50 μM). In contrast, there was no detectable PARP cleavage in the Apo2L/TRAIL or CPT-11 treatments alone. These data indicate that caspase-3 is primarily activated during apoptosis induced by Apo2L/TRAIL plus CPT-11.

Caspase-8 is an apical caspase activated in the cell-extrinsic, receptor-mediated apoptosis pathway (16). We next assessed whether caspase-8 was activated in the combination treatment by Western blot analysis. The results indicate that the levels of procaspase-8 were decreased by 2–4 h in the Apo2L/TRAIL plus CPT-11 treatment as compared with single treatments (Fig. 2*E*), indicative of its cleavage and activation. Bid is a proapoptotic BH3-only Bcl-2 family protein reported to be activated by caspase-8 during treatment with many agents (35), including Apo2L/TRAIL (23, 42). The appearance of the M_r 15,000 cleavage derivative of Bid as early as 4 h after the combination treatment, which was prevented by zVAD-fmk, indicated Bid activation (Fig. 2*E*). Activated Bid is known to further activate the multidomain (BH123) proapoptotic Bcl-2 family proteins Bax and Bak, thus providing the cross-link between the cell-extrinsic and cell-intrinsic pathways of apoptosis (32, 35). Bax protein levels were indeed increased during the combination treatment, with zVAD-fmk having little effect at 4 h (Fig. 3*C*). These results, in conjunction with those above demonstrating that the combined treatment also induced caspase-3 and -7 activation, indicate that the Apo2L/TRAIL and CPT-11 combination treatment initiates apoptosis through both the cell-extrinsic and cell-intrinsic pathways.

Increased *bax* Gene Expression and Activation. To determine the molecular mechanism for expression of *bax* and related apoptotic genes, the steady-state levels of their mRNAs were examined by the multiprobe RPA. CPT-11 treatment for 8 h led to increased *bax* expression as compared with Apo2L/TRAIL alone. In the combination treatment, *bax* expression was further increased (3.3- *versus* 2-fold; Fig. 3*A*). However, the expression of *bcl-xL* was down-regulated. As expected, CPT-11 also increased the expression levels of a prototypic stress response gene, *p21/waf1*, with its levels being further increased after the combination treatment (Fig. 3*A*). Interestingly, another stress response gene, *gadd45*, was activated 3-fold by the combination treatment (Fig. 3*A*).

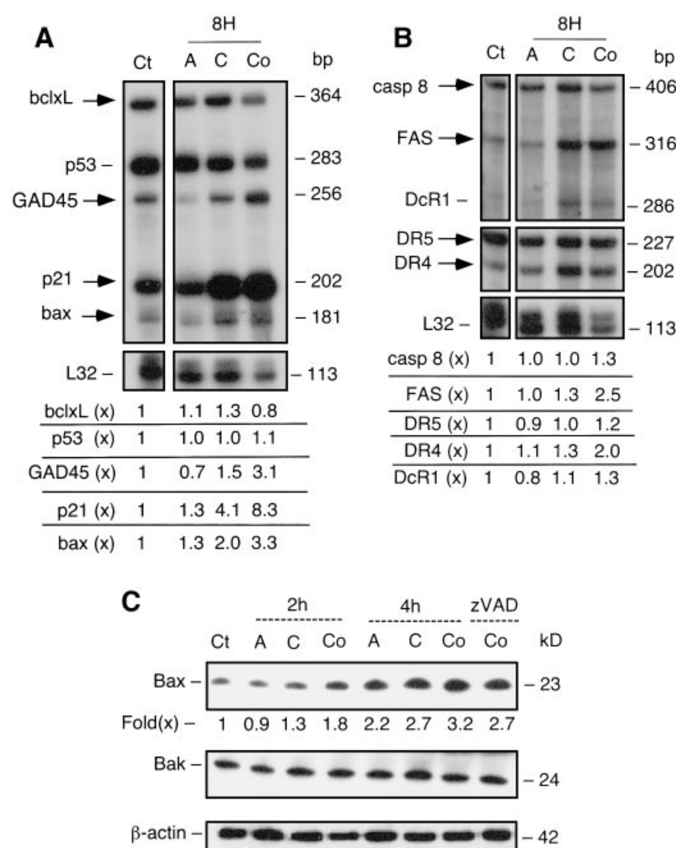

Fig. 3. Expression of apoptotic regulators in Apo2L/TRAIL plus CPT-11-treated C4-2 cells. Cells were treated with Apo2L/TRAIL (*A*), CPT-11 (*C*), and the combination (*Co*) for 8 h, and steady-state mRNA expression was analyzed by RPA using hStress-1 (*A*), and hAPO3c (*B*) multiprobe templates. The mRNA levels were shown as fold change (*x*) relative to untreated control cells (*Ct*), and the size of the protected RNA products is indicated *below* and to the *right*, respectively. All data represent values obtained by normalizing levels of mRNAs of treated cells to those of untreated control cells and the L32 control, using the Image Quant Program. *C*, induction of Bax protein by Apo2L/TRAIL and CPT-11 in C4-2 cells. Cells were treated with Apo2L/TRAIL, CPT-11, and the combination for 2 and 4 h and subjected to Western blotting for Bax, Bak, and β-actin, as a control.

Bax is activated by a change in its conformation, leading to exposure of an occluded NH_2-terminal sequence (47). Bax activation was examined by immunocytochemistry using an antibody against the activated Bax. The combination treatment caused Bax immunoreactivity, with active Bax condensed foci being formed at the mitochondrial sites visualized by a mitochondria-specific dye (MitoTracker Red; Fig. 4*A*). Active Bax also caused the release of cytochrome *c* from damaged mitochondria. This was evidenced by the diffusion of cytochrome *c* from mitochondrial sites (Fig. 4*B*). Thus, these results indicate that Bax may be activated by Bid to induce apoptosis, with Bid thus facilitating a cross-talk between the cell-extrinsic and cell-intrinsic pathways of apoptosis. Therefore, the Apo2L/TRAIL-mediated cell-extrinsic pathway of apoptosis is not sufficient to induce apoptosis in C4-2 cells, requiring an amplification step through the cell-intrinsic signaling pathway mediated by Bid.

We next examined expression levels of several components of the

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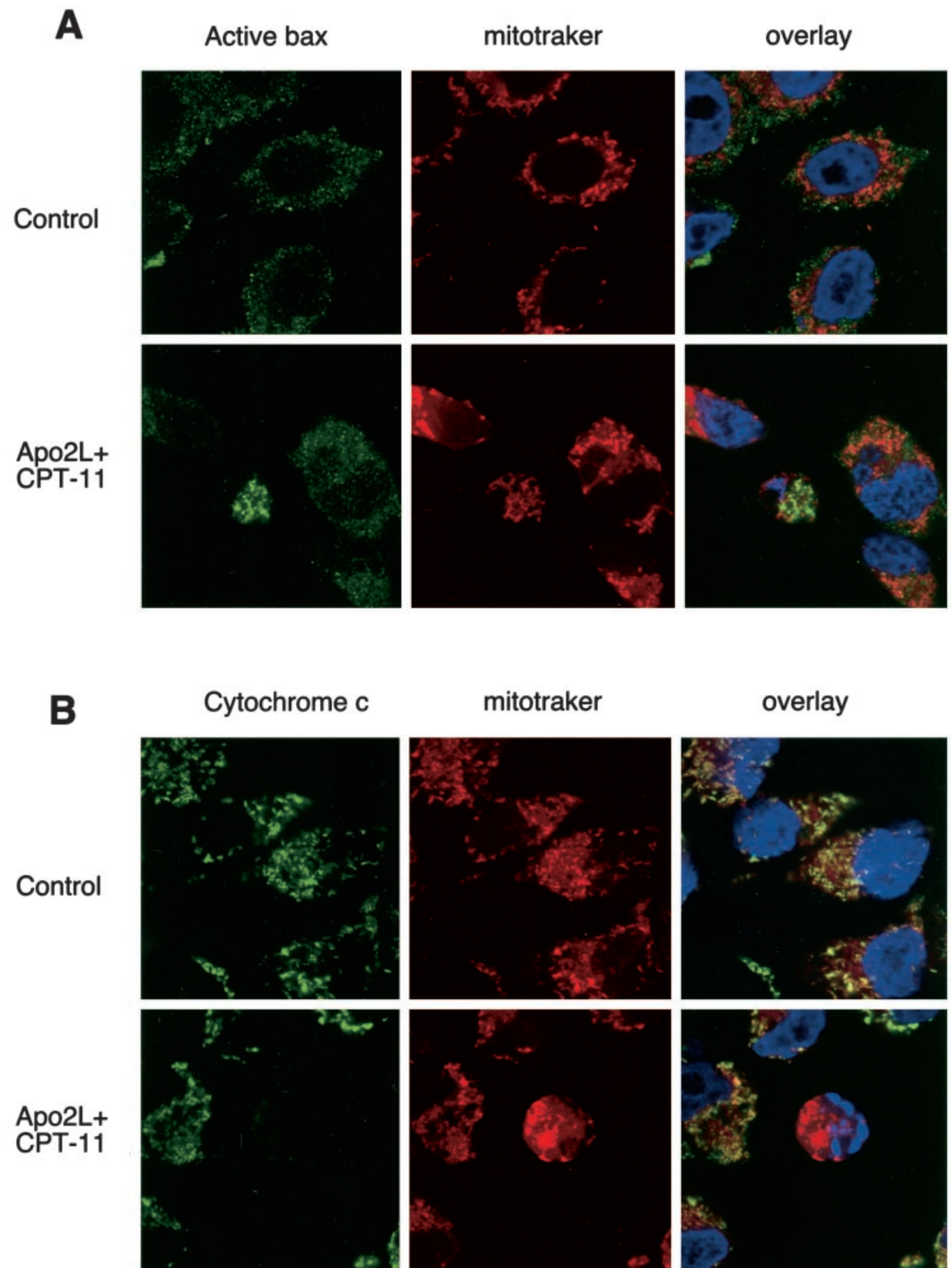

Fig. 4. Activation of Bax and release of cytochrome *c* from mitochondria by Apo2L/TRAIL and CPT-11. Cells were treated with Apo2L/TRAIL plus CPT-11 for 4 h and then immunostained with antibody against (*A*) active Bax and (*B*) cytochrome *c*, and the primary antibodies were detected by Alexa Fluor 488-conjugated secondary antibodies. The mitochondria were stained with the mitochondria-specific dye MitoTracker Red, and cells were mounted with 4′,6-diamidino-2-phenylindole (DAPI) containing Vectashield (representative areas were photographed using a ×63 objective).

receptor-mediated pathway of apoptosis. CPT-11 increased the mRNA levels of the Apo2L/TRAIL DRs (*DR4* and *DR5*) and the *Fas* receptor, as well as caused a slight increase in *caspase-8*. These data indicate that even though CPT-11 treatment up-regulated the expression of *DR4* and *DR5*, this change was not sufficient to initiate apoptosis. Combining CPT-11 treatment with Apo2L/TRAIL led to a further increase of their expression levels (Fig. 3*B*). These results indicate that the Apo2L/TRAIL plus CPT-11 treatment induces apoptosis through modulation of expression levels of several Bcl-2 family genes and their products as well as components of DR signaling. Importantly, even though changes in expression level of individual genes are rather modest, their combined effect is likely to be critical.

Effect of ΔDR5 Expression and *bax* Gene Ablation. To examine the contribution of DR5 to apoptosis, we stably expressed a FLAG epitope-tagged dominant-negative ΔDR5 into C4-2 cells. Treatment with Apo2L plus CPT-11 for 48 h induced 58% killing in C4-2 cells, whereas it induced 46% killing in ΔDR5-expressing cells, as measured by the MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (Ref. 45; Fig. 5*A*). This result indicates that inactivation of DR5 by ΔDR5 (Fig. 5*B*) caused partial resistance to this treatment. In addition, PARP cleavage induced by the combination treatment in ΔDR5-expressing cells was greatly diminished as compared with C4-2 cells (Fig. 5*D*), indicating that the cell-extrinsic pathway is necessary for apoptosis.

To determine the effect of Bax on apoptosis, we transfected C4-2 cells transiently with siRNA (*bax*). *bax* gene expression and the encoded protein levels were dramatically down-regulated at 24–48 h after transfection, as determined by Western blotting (Fig. 5*C*). Down-regulation of *bax* by RNA interference in C4-2 cells significantly prevented apoptosis (37% killing) and PARP cleavage (Fig. 5, *A* and *E*, respectively). Interestingly, transfection of siRNA (*bax*) in stably ΔDR5-expressing C4-2 cells led to complete ablation of PARP cleavage and apoptosis (Fig. 5, *E* and *A*, respectively), indicating the

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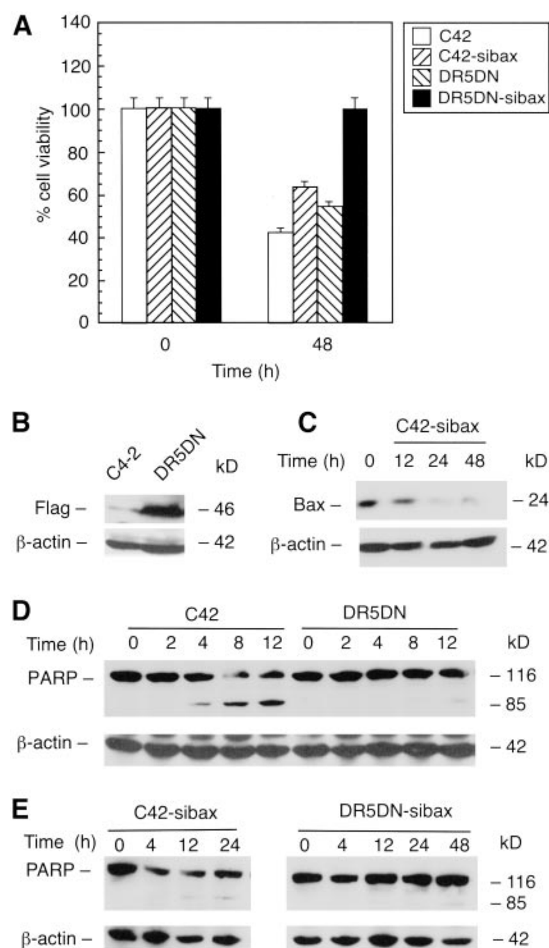

Fig. 5. The apoptotic effect of Apo2L/TRAIL plus CPT-11 is DR5 and Bax dependent. C4-2 vector control, ΔDR5-FLAG-containing C4-2 cells, silencer siRNA (*bax*)-transfected C4-2 cells, and silencer siRNA (*bax*)-transfected ΔDR5-FLAG-expressing C4-2 cells were treated with Apo2L/TRAIL plus CPT-11. *A*, cell viability was assessed by MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay after 48 h and expressed as the percentage of the control untreated cells. The data shown (mean ± SD) are from at least three independent experiments. *B*, expression of ΔDR5 was determined by Western blotting using a FLAG antibody and β-actin as a loading control. *C*, silencing of *bax* gene was determined by measuring expression of Bax protein at different time points after transfection with silencer siRNA (*bax*) by Western blotting using Bax antibody. *D* and *E*, after treating control C4-2, DR5DN, C4-2-sibax, and DR5DN-sibax cells with Apo2L/TRAIL plus CPT-11 for different time periods, lysates were analyzed for immunoblotting with an anti-PARP antibody.

essential role of both receptor-mediated and mitochondrial pathways of apoptosis induced by Apo2L/TRAIL plus CPT-11.

Antitumor Activity of Apo2L/TRAIL with CPT-11 *in Vivo*. Next, we tested the effect of Apo2L/TRAIL and CPT-11 on the progression of established C4-2 tumors. We treated mice bearing s.c. tumors (~100 mm^3) with vehicle control (TBS for 5 days), Apo2L/TRAIL (60 mg/kg/day for 5 days, on days 13–17), CPT-11 (20 mg/kg/day for 3 days, on days 12, 16, and 20), and the combination. The concentration of 20 mg of CPT-11/kg/day was determined from dose-response experiments to represent a suboptimal dose resulting in a minimal toxic effect (Fig. 6*A*). Treatment with Apo2L/TRAIL alone or CPT-11 had a minor effect on the overall growth of C4-2 tumors as compared with the vehicle control. In contrast, further tumor growth in the mice treated with Apo2L/TRAIL plus CPT-11 was prevented, and the tumor volume remained the same up to 25 days after inoculation of tumor cells.

One group of mice that received the combination treatment was given a second round of Apo2L/TRAIL injections for 5 consecutive days (days 28–32). In these animals, tumor size remained constant or diminished, with an inhibition of tumor growth at day 42 of 85% as compared with control. Most significantly, one-third of the animals (two of six) showed complete tumor elimination. The other four animals in this group showed little increase in tumor volume by day 42, as compared with their initial tumor volume on day 12 (Fig. 6*B*). In contrast, the tumors that received the combination treatment without the second round of Apo2L/TRAIL injection began to increase in size, with an inhibition of tumor growth of 57.5% as compared with the control at day 42. Moreover, there were no animals in which the tumors were eliminated. These data indicate that Apo2L/TRAIL shows significant antitumor activity when combined with CPT-11 and, led to complete remission of the established tumors in some of the animals.

To examine apoptosis *in vivo*, we sacrificed the mice in two sets: at day 13 and at day 17. By immunohistochemical analysis, we observed enhanced active caspase-3 and active caspase-7 expression in the combination treatment in both tumor sets (Fig. 7). However, similar to the *in vitro* culture experiments, staining for caspase-7 was much weaker and was seen in fewer cells. Immunoblot analyses further indicated enhanced cleavage of procaspase-8, procaspase-9, and a complete cleavage of the caspase-3 substrate PARP, in the combined treatment by immunoblot analysis of tumor homogenates taken from tumors at day 13 (Fig. 8*A*). This indicates apoptosis induction through activation of multiple caspases by the Apo2L/TRAIL plus CPT-11 treatment.

Examination of Bax levels indicated Bax induction in day 13 tumors, but not day 17 tumors. Nevertheless, expression levels of Bax were comparable in the two tumors. In contrast, Bak protein levels were virtually undetectable in all the day 13 tumors or those that were isolated at day 17 but had not received any treatment. Strikingly, Bak levels were induced by CPT-11 and induced even more dramatically by the combined treatment in tumors isolated at day 17 (Fig. 8*B*). Interestingly, the mRNA expressions for the antiapoptotic Bcl-2 family members *bcl-ω* and *bcl-xL* were lower. In addition, mRNA levels for the proapoptotic *bcl-xS* were increased in the combined treatment as compared with the single treatments (Fig. 8*C*). The day 17 tumors that received the combination treatment showed an abundant expression of Bak, whereas the day 13 tumors or those from day 17 that had not received any treatment, had no detectable levels of Bak by histology (Fig. 7). Taken together, these results indicate that the Bcl-2 family proteins play an important role in induction of apoptosis by the combination treatment in established tumor xenografts. Moreover, they indicate that, even in similar cells, under different biological conditions, different Bcl-2 family members may be responsible for inducing apoptosis.

DISCUSSION

In the present study, we evaluated the antitumor activity of Apo2L/TRAIL plus CPT-11 treatment *in vitro* and *in vivo* in C4-2 human prostate cancer cells. The combination of Apo2L/TRAIL and CPT-11 activated both the cell-extrinsic and cell-intrinsic pathways of apoptosis by inducing expression and activation of Bcl-2 family proteins and several caspases. We found that this combination treatment triggered apoptosis in C4-2 cells by cleavage and activation of caspase-8 and -3, which were blocked by the pan-caspase inhibitor zVAD-fmk. Caspase-7 was also activated, but only weakly as compared with caspase-3. Induced expression and activation of Bax and down-regulation of Bcl-xL were also observed and are likely to have played an important role. Importantly, the combination treatment also led to regression of tumor growth and induction of apoptosis in C4-2 tumors

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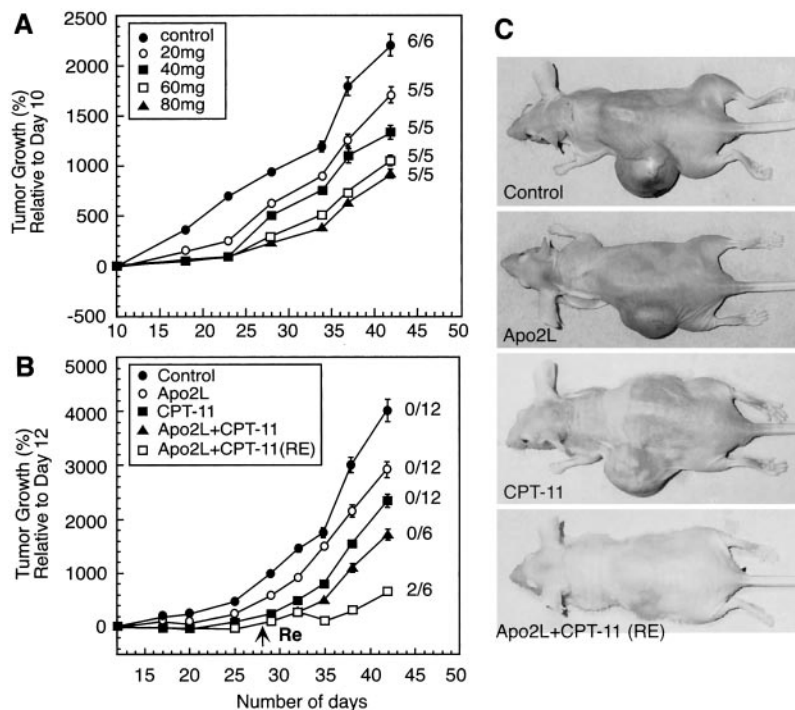

Fig. 6. Effect of the combination of Apo2L/TRAIL and CPT-11 on growth of established C4-2 tumors. Athymic (*nu/nu*) mice received s.c. injection with 3×10^6 C4-2 cells. At day 12 after tumor implantation, tumor-positive animals were randomly sorted into treatment groups, and the average tumor size for each group was determined. The value for each group was set to 0%, and all subsequent changes in tumor size for each group were expressed as a percentage change in comparison with the starting tumor mass. *A*, dose response of CPT-11 (20–80 mg/kg/day) on tumor growth. *B*, treatments of day 12 established C4-2 tumor were with TBS, 60 mg/kg/day Apo2L/TRAIL, 20 mg/kg/day CPT-11, and the combination of 60 mg/kg/day Apo2L/TRAIL plus 20 mg/kg/day CPT-11. Apo2L/TRAIL was administered by i.p. injection from days 13–17 (5 consecutive days). CPT-11 was administered by i.p. injection on days 12, 16, and 20 after tumor implantation. One group of mice from the combination-treated group received another course of injection of Apo2L/TRAIL at days 28–32 [*Apo2L + CPT-11 (RE)*]. Tumor size was determined twice a week, and the mean tumor size of tumor-bearing mice was shown. The number of tumor-free animals/number of total animals per treatment group at the end of experiment is shown. *C*, the photograph of tumor-bearing mice was taken at the end of the experiment.

xenografted in mice by inducing cleavage of caspase-8, -3, -7, and -9 and PARP and up-regulation of the Bax homologue, Bak.

Apoptosis induced by cell surface receptors (DR4 and DR5) is mediated by the recruitment and activation of an apical caspase (either caspase-8 or -10) to the receptor, which subsequently activates downstream executioner caspases (caspase-3, -7, or -6). In several human prostate cancer cell lines (LNCaP, PC3, and DU145), different caspases were activated *in vitro* as well as *in vivo* during piperazine-

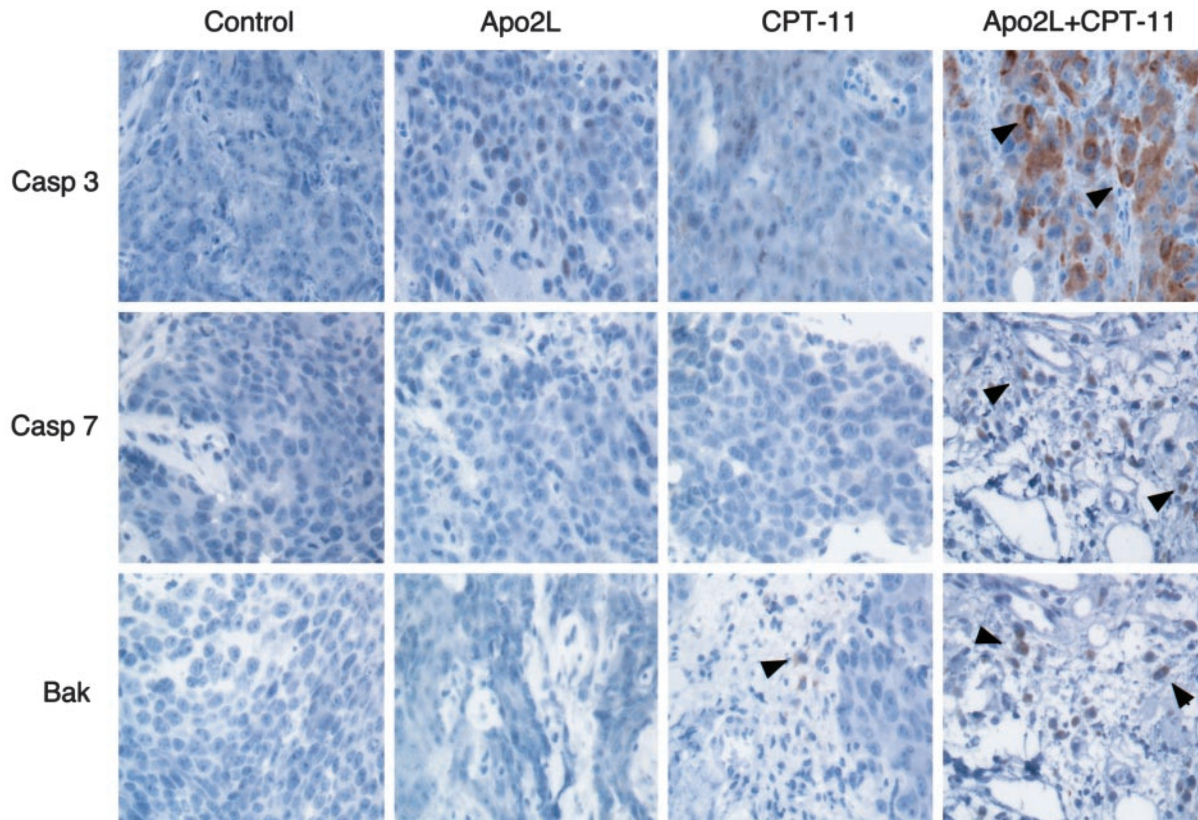

Fig. 7. Immunohistochemical analysis of tumor sections with active caspase-3, active caspase-7, and Bak antibodies. Parrafin-embedded tumor sections from Apo2L/TRAIL, CPT-11, and the combination treatment were stained with active caspase-3, active caspase-7, and Bak antibodies using Ventana ES Autostainer and 3,3′-diaminobenzidine kit by immunoperoxidase staining. Sections were examined by light microscopy, and representative areas were photographed using a ×20 objective. *Arrowheads* indicate immunostained cells.

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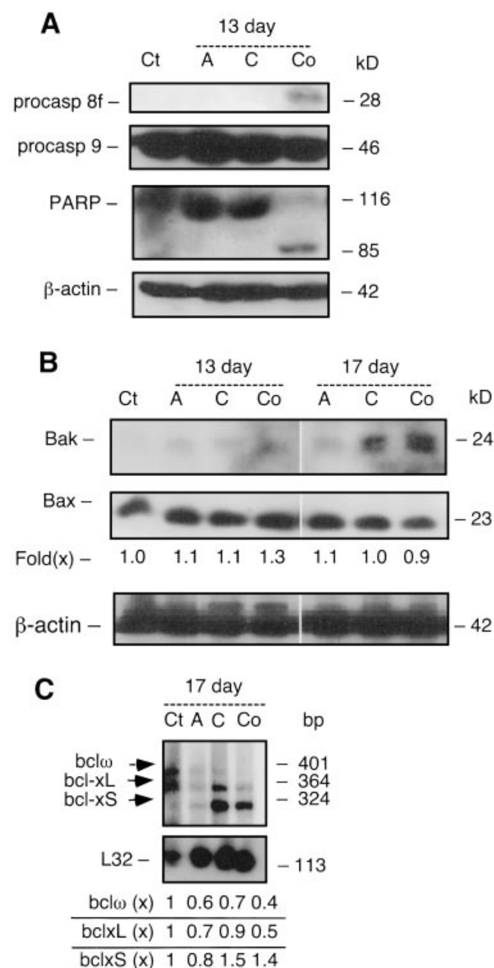

Fig. 8. *In vivo* tumor control by Apo2L/TRAIL with CPT-11. Nude mice with s.c. C4-2 tumors were treated with vehicle control, Apo2L/TRAIL (60 mg/kg/day for 13–17 days), CPT-11 (20 mg/kg/day for 12, 16, and 20 days), and the combination by i.p. injection. *A*, tissues were collected on day 13 and assayed for different caspases and PARP cleavage by Western blotting. For the analysis of Bcl-2-family members, tissues were collected on days 13 and 17 and analyzed for protein expression (Bax and Bak) and RNA for different Bcl-2 family members by Western blotting (*B*) and RPA using the hAPO2b multiprobe template (*C*), respectively.

induced apoptosis (48). In our study, the combination of Apo2L/TRAIL plus CPT-11 induced caspase-8, caspase-3, and PARP cleavage similar to previous reports (15, 49). A recent clinical report revealed that higher expression of Bid correlated with longer recurrence-free survival in men with locally advanced (T_3 stage) prostate cancer. Overexpression of Bid by gene transfection resulted in increased sensitivity to apoptosis induction by a ribonucleotide reductase inhibitor drug (50). In our study, an enhanced expression of Bid was found in the combination treatment. Moreover, previous studies have shown that Bid activation mediates the amplification of Apo2L/TRAIL-mediated apoptosis through the mitochondria-mediated pathway through activation of Bax and/or Bak. Activated Bax translocates to the mitochondria, causing cytochrome *c* release and, subsequently, activation of caspase-9 and -3 (34). Our results are consistent with the notion that Bid is cleaved and activated by active caspase-8 and that truncated Bid translocates to the mitochondria to induce the cell-intrinsic pathway of apoptosis by activating and inducing Bax expression and releasing cytochrome *c* from the mitochondria.

Recently, it has been shown that Bax plays an important role in apoptosis. Gene knockout of Bax and/or Bak in mouse hepatocytes showed that these proteins were necessary, although mutually redundant, for DR engagement of mitochondria (51, 52). However, somatic knockout of Bax in human HCT116 colon cancer cells indicated an absolute requirement for Bax in DR engagement of the mitochondria (53, 54). Both Bax+/− and Bax−/− cells expressed Bak, but this was not sufficient to substitute for Bax, perhaps due to its relatively low levels of expression in most human cells. Mismatch repair-deficient tumors can acquire resistance to Apo2L/TRAIL through mutational inactivation of Bax (53). However, pretreatment with CPT-11 induced Bak and rescued sensitivity to Apo2L/TRAIL (53). Remarkable in our study was the sharp contrast between Bak expression *in vitro* and *in vivo.* Whereas there was abundant expression of Bak *in vitro*, no Bak was detectable *in vivo* in any of the day 13 tumors, treated or untreated, or in the untreated day 17 tumors, as determined by immunohistochemistry or immunoblotting. Strikingly, there was a strong induction of Bak in the day 17 tumors that had received the combination treatment.

The ratio of Bax homodimers:Bax/Bcl-2 heterodimers has been considered an apoptotic checkpoint. When Bcl-2 is in excess, apoptosis is inhibited. However, if Bax levels increase in response to a death signal, the cell is pushed toward death. Bax forms heterodimers with Bcl-2, effectively antagonizing Bcl-2 function and thus promoting apoptosis (36). In our study, the Apo2L/TRAIL plus CPT-11 treatment induced apoptosis in C4-2 cells by down-regulating Bcl-xL and up-regulating Bax protein expression *in vitro*, with sequential activation of Bax and Bak *in vivo.* By overexpression of Bcl-2, much of the Bax in Bax/Bcl-2 heterodimers was reported to result in blocking the death signal in prostate cancer cells (55). By down-regulation of both Bcl-2 and Bcl-xL using antisense oligonucleotides, it was found that LNCaP and PC3 cells became more sensitive to different chemotherapeutic agents (56). Taxol induced apoptosis in PC3 and LNCaP cells by down-regulation of Bcl-xL, whereas estramustine-induced apoptosis in LNCaP was associated with up-regulation of Bak (57).

Several mechanisms have been proposed to regulate Bcl-2 family protein functions. One of them is a conformational change (58–60). Cell damage promotes changes in the NH_2 termini of several pro-apoptotic Bcl-2 family proteins. A conformational change in the Bak NH_2 terminus and/or the loss of an NH_2 terminal-binding protein was associated with the activation of Bak in response to many DNA-damaging insults (59). A conformational change due to a change in the NH_2 terminus of Bax has been reported in different cell types after treating cells with DNA-damaging agents (59–62). In Hela cells, this NH_2 terminal change in Bax depended on the binding of uncleaved Bid and was followed by Bax oligomerization and insertion into the outer mitochondrial membrane (63). Our study suggests that Apo2L/TRAIL plus CPT-11 treatment induced a conformational change in Bax by Bid, resulting in the activation of Bax and translocation of active Bax to the mitochondria, as evidenced by the colocalization of active Bax and mitochondria by immunocytochemical staining. Our data also show that the translocation of active Bax to the mitochondria is associated with the release of cytochrome *c* from the mitochondria. Similarly, translocation of Bax and oligomerization of Bax and Bak may be responsible for apoptosis activation *in vivo.* Moreover, down-regulation of Bax by siRNA in C4-2 cells significantly prevented PARP cleavage and apoptosis. Thus activation of the mitochondrial pathway also occurs during Apo2L/TRAIL plus CPT-11-induced apoptosis in C4-2 cells.

Previous work has indicated that ionizing radiation (23, 24), etoposide (44, 64), or CDDP (44) sensitizes tumor cells to Apo2L/TRAIL-mediated apoptosis by up-regulating the Apo2L/TRAIL receptor DR5. In the present study, the combination treatment significantly induced the expression of DR4 and, to a lesser extent, that of DR5. Although the combination treatment also led to increased levels of DcR1, this might not be sufficient to overcome the effect of

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the increased levels of DR4 and DR5. Therefore, the induction of these proapoptotic molecules is consistent with additional evidence of apoptosis by the combination treatment in C4-2 cells. Moreover, dominant negative Apo2L/TRAIL receptor ΔDR5 was able to partially block Apo2L/TRAIL plus CPT-11-mediated apoptosis and to delay and decrease the amount of PARP cleavage. Transfection with siRNA (*bax*) in ΔDR5-expressing C4-2 cells completely prevented PARP cleavage and apoptosis by the combination treatment, indicating the role of both receptor-mediated and mitochondrial pathways in apoptosis.

Apo2L/TRAIL has been shown to exert potent cytotoxic activity against many tumor cell lines, but not most normal cells (4, 5, 11, 17). In animal models, Apo2L/TRAIL can also suppress tumor growth *in vivo* without affecting normal tissues (13, 17). In this study, we carried out a detailed investigation of the antitumor effects of Apo2L/TRAIL in combination with the chemotherapeutic agent CPT-11. A dose-response study of CPT-11 indicated that a higher dose of 60–80 mg/kg/dose was toxic, thus requiring a lower dose of CPT-11 (20 mg/kg/dose) to achieve therapeutic benefit in combination with Apo2L/TRAIL. The antitumor activity of Apo2L/TRAIL could be greatly augmented by its use in combination with this lower dose of CPT-11. Treatment with Apo2L/TRAIL alone had only a minimal effect on tumor growth, consistent with the resistance of C4-2 cells to Apo2L/TRAIL *in vitro.* Treatment with CPT-11 alone and the combination of Apo2L/TRAIL and CPT-11 slowed down the tumor growth significantly, with tumor volume remaining constant up to the 25th day. The tumors in the CPT-11 and combination treatment groups that did not receive a second course of Apo2L/TRAIL injection started to regrow, with no animal becoming tumor free. Importantly, treatment of mice that received the combination of Apo2L/TRAIL and CPT-11 with a second course of Apo2L/TRAIL injection not only caused the greatest tumor suppression and regression as compared with the other treatment groups but also resulted in the complete elimination of tumors in two of six animals. Apo2L/TRAIL, CPT-11 alone, or the combination did not produce any substantial, observable toxic effects at the doses indicated because overall body weight of mice in all treatments were unchanged (data not shown).

In summary, we delineated the mechanism of the synergistic cytotoxic effect of Apo2L/TRAIL and CPT-11 toward apoptosis of C4-2 prostate cancer cells and tumors. We identified several caspases and Bcl-2 family proteins including activation of Bax and induction of Bak, which play an important role in inducing Apo2L/TRAIL plus CPT-11-mediated apoptosis. This combination treatment needed both cell-extrinsic and cell-intrinsic pathways to induce apoptosis in C4-2 cells because inactivation of DR5 and Bax completely prevented cell death. There was a striking difference between expression and activation of apoptotic regulators *in vitro* and *in vivo*, with the most significant difference being Bak expression, indicating that different molecular means may be used by the same cells under different biological conditions to activate apoptosis. Our study suggests that the combination of Apo2L/TRAIL plus CPT-11 exerts antitumor activity both *in vitro* and *in vivo.* Several chemotherapeutic agents have been shown to have a synergistic cytotoxic effect with Apo2L/TRAIL (17, 26, 65), indicating that a combination therapy using Apo2L/TRAIL with CPT-11 is likely to be widely applicable and may become a potentially promising, novel anti-prostate cancer therapeutic modality.

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